Clinical Significance of Plasma Bone-specific Alkaline Phosphatase Measurement and the Alkaline Phosphatase Isozymes Expression in Osteosarcoma

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Abstract

Osteosarcoma is one of the most commonly occurred primary malignant bone tumor affecting children and young adults. With the improvement of chemotherapeutic regimes, adjuvant chemotherapy and surgical technique, both the successful rate of long term survival and the quality of life of the patients are improved. However, the need of a plasma biochemical marker to monitor the response of treatment also becomes more important

In this research, we tested the possibility of using plasma bone-specific alkaline phosphatase (BALP) as a tumor marker for osteosarcoma. Firstly, we established the normal reference value of plasma BALP level in local Chinese population. The normal reference values of plasma BALP decline across the age, with normal range of 73.6 U/L to 217.7 U/L in age below 12 (N1); 21.7 U/L to 136.8 U/L in age between 12 to 16 (N2) and 8.9 U/L to 59.5 U/L in age above 16 (N3).

We found that plasma BALP activity of the osteosarcoma patients at admission (BALP-adm) is significantly higher than the normal groups ($p<0.005$). When age-match comparison is made, we found that at least in two age groups, namely age between 12 to 16 and age above 16, the plasma BALP-adm is significantly higher than the normal group ($p<0.01$).

Moreover, patients with poor clinical outcome had a significantly higher plasma BALP-adm compared to other patients. Patients who died of disease within one, two and three years have a significantly higher plasma BALP-adm than patients who remained disease-free ($p<0.05$). In addition, plasma BALP-adm can reflect the chance of local recurrence after the surgical treatment. Patients who experience local relapse have a significantly higher plasma BALP-adm values than others ($p <0.001$).

Of most clinical importance, plasma BALP level reflects the patients' responsiveness in the treatment processes , therefore allowing the monitoring of the disease. The change of plasma BALP level during the chemotherapy correlate well to the responsiveness of the patients toward the chemotherapy. Reflected by the degree of tumor necrosis, patients with their plasma BALP level drops back within the normal range have a significantly higher tumor necrosis ($p < 0.05$). Moreover, the change of plasma BALP level correlates well to the clinical observation of the patients. Except in patients with incomplete removal of the tumor (including metastases), the plasma BALP level always drops back to low level after the operation and elevated during recurrence.

Ectopic expression of placental ALP was found in human osteosarcoma cell line U-2 OS by biochemical differentiation, isoelectric focusing separation and immunohistostaining. Comparing with the ALP extracted from Sa OS-2 cell which contains solely BALP (Farley *et al*, 1989), the ALP expressed in U-2 OS cells is thermostable, sensitive to L-phenylalanine inhibition but insensitive to levamisole inhibition and can bind to monoclonal antibody against human placental ALP. However, no such ectopic expression of placental ALP could be found in our 14 osteosarcoma patients' plasma and tissue extracts and 7 osteosarcoma tissue sections.

Chapter One: Introduction

1.1 Osteosarcoma

1.1.1 Definition

Among the malignant tumors of the skeleton in children and adolescents, osteosarcoma are particularly important because of their high incidence and a great chance of successful treatment using chemotherapy in combination with surgery.

Osteosarcoma is a new term most authors used to replace the term 'osteogenic sarcoma'. It is defined by World Health Organization (WHO) as "*a malignant tumor characterized by the direct formation of bone or osteoid by the proliferation tumor cells*" (Schajowicz, 1994).

This definition is based on the usually readily recognizable product of this neoplasm. Nevertheless, even small foci of tumor osteoid within a predominantly chondroblastic, fibroblastic, histolytic of osteoclastic tumor give the diagnosis of osteosarcoma (Figure 1.1).

1.1.2 Incidence, Geographic patterns of distribution and epidemiological consideration

Excluding plasma cell myeloma, osteosarcoma is the most frequent primary malignant bone tumor. In United State, there are 600 to 800 new cases per year (Himelstein & Dormans, 1996). However, the true incidence of bone tumor in general, osteosarcoma in particular, is difficult to estimate because the population-based tumor registries record too few bone cancer cases to permit separation into the various types.

In 1971, the Swedish National Cancer Registry reported an annual incidence of 0.28 cases per 100,000 people with no geographic difference (Larsson & Lorentzon, 1974). However, studies in Malaysia shows both geographic and radical difference in incidence rate of osteosarcoma with 0.11 case per 100,000 people in Malays and 0.23 cases in Chinese and Indian population respectively, and with higher incidence in urban area (Bovill *et al.*, 1975).

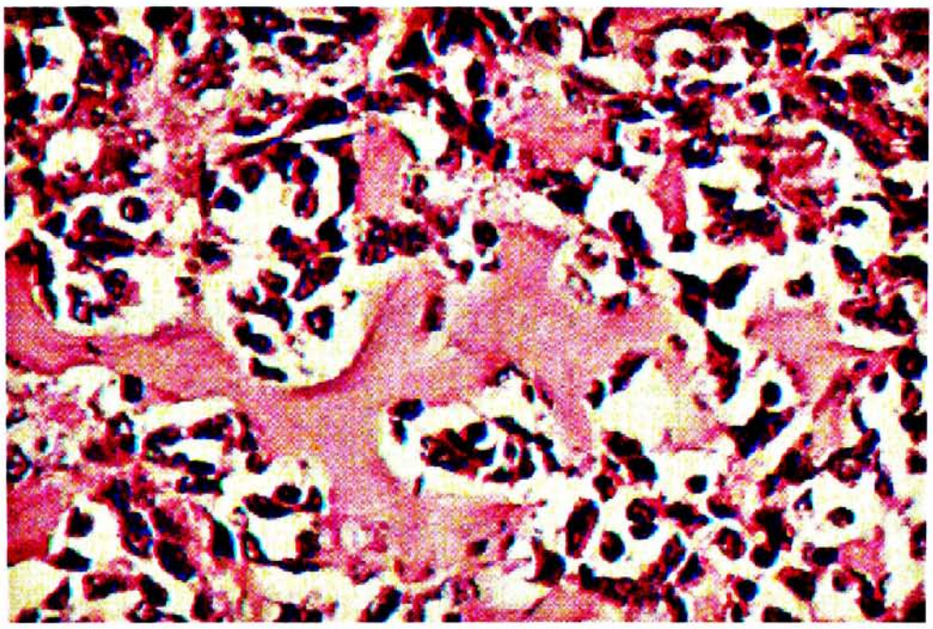


Figure 1.1 Osteosarcoma with marked calcified osteoid production (Hematoxylin-eosin stain, Magnification ×100)

1.1.3 Age, Sex and Sites

Osteosarcoma can occur at any age, although it is chiefly an affliction of the young. There is evidence that age, sex and the anatomical site of osteosarcoma correlate well with the periods of rapid growth.

The age and sex distribution of osteosarcoma in Memorial Sloan-Kettering Cancer Center is shown in Figure 1.2. (Huvos, 1991). The total number of cases collected is 685. The tumor occurs more often in males, with 402 males patients (58.6%) and 283 females (41.3%), which agrees with the general male predominance in most other studies (Dahlin & Unni, 1986; Campanacci, 1990; Schajowicz, 1994). The higher incidence of tumors in men may be related to the longer period of skeletal growth and the additional volume of bone produced compare with the female. It is also notable that osteosarcoma tends to start at an earlier age in females than in males and patients affected by osteosarcoma have been found to be taller than their peers in the corresponding age group and also with high plasma somatomedin levels (McMaster, 1977). All these data show that the age period with highest appearance of osteosarcoma is coincided with the periods of sudden burst of skeletal growth.

Approximately 10 percent of all osteosarcoma arise in patients older than 60, causing part of second "hump" in bimodal age distribution curve of the patients (Huvos, 1986,1991). Unlike those younger patients, with 97% of osteosarcoma are newly arise, osteosarcoma in this group of patients were more frequently (56%) secondary to other bony conditions, such as Paget's disease, or followed irradiation.

In majority of the patients, especially the younger one, osteosarcoma shows a preference for the long bones. Its occurrence has a predilection for the metaphyseal areas of the lower end of the femur, the upper end of tibia and less frequently, the upper end of the humerus, that is, close to the most actively growing epiphyses. Approximately one-half of all tumors affected

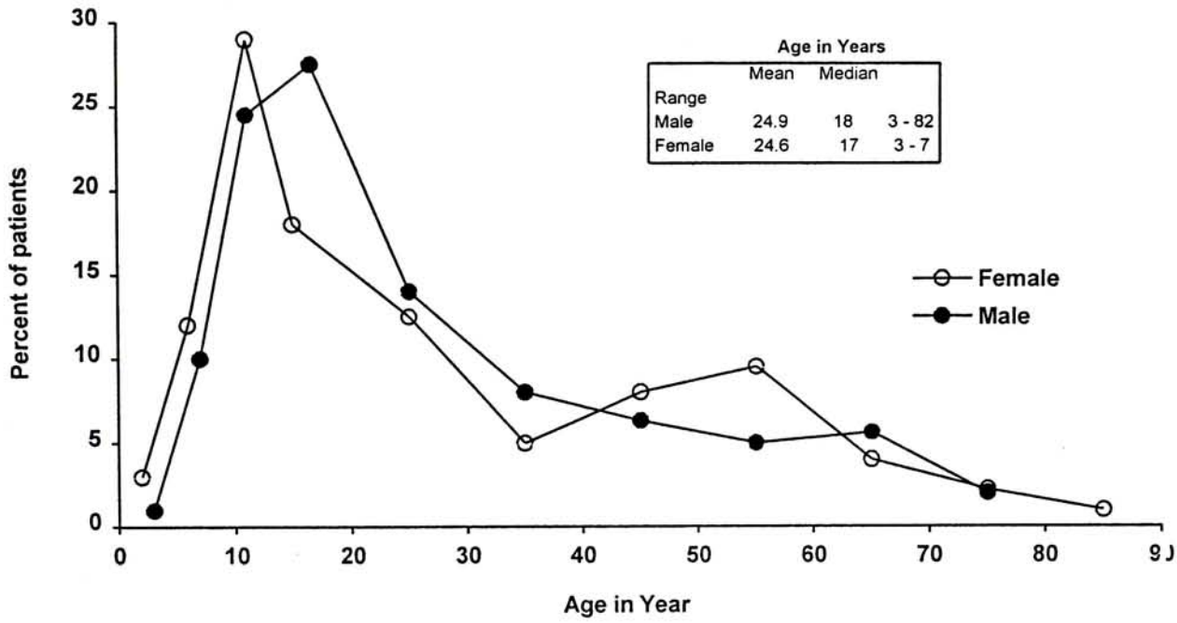


Figure 1.2 Age and Sex distribution in 1095 patients with osteosarcoma diagnosed in Mermorial Sloon-Kettering Cancer Centre 1921 through 1979 (Huvos, 1991).

the knee region, and the femur was the most common bone involved (Figure 1.3).

In contrast to osteosarcoma in children and adolescents, the knee joint region is involved in only 14.5% of the older patients. In this latter group, the axial skeleton was the most commonly affected (27%), in addition to the craniofacial bones and extraskeletal sites (Huvos, 1986). In conclusion, before the cessation of the growth period, the long bones are the most frequently involved in osteosarcoma. After this growth period, the long and the flat bones are about equally affected (Huvos, 1991).

1.1.4 Type and Grade

Osteosarcoma covers a wide spectrum of lesion with distinct clinical and pathologic features, associated with different biological behavior. Two fundamental groups can be clearly distinguished namely, the central (medullary) and the surface (peripheral) osteosarcoma, depending on the site of the lesions (Schajowicz, 1983).

Since the diagnosis of osteosarcoma is only based on the detection of tumor bone or osteoid, even when the predominant tissue is cartilaginous or fibroblastic, the lesion will still be considered as osteosarcoma. Several researchers tried to subdivide the osteosarcoma into different subtypes in order to give a more precise description of the lesions. Dahlin (1978) subdivided osteosarcoma into three categories, depending on the predominant differentiation of the tumor cells. They are the "osteoblastic osteosarcoma" with large amount of tumor bone and osteoid production; "chondroblastic osteosarcoma" in which the tumor cells show predominant differentiation toward cartilage; and "fibroblastic osteosarcoma", in which tumor cells show predominant spindle-cell pattern similar to that seen in fibrosarcoma.

A system of staging the musculosketetal neoplasm was adapted by the American Joint Committee Task Force on Bone Tumors in 1985 and proposed by them to the international Union Against Cancer for international usage

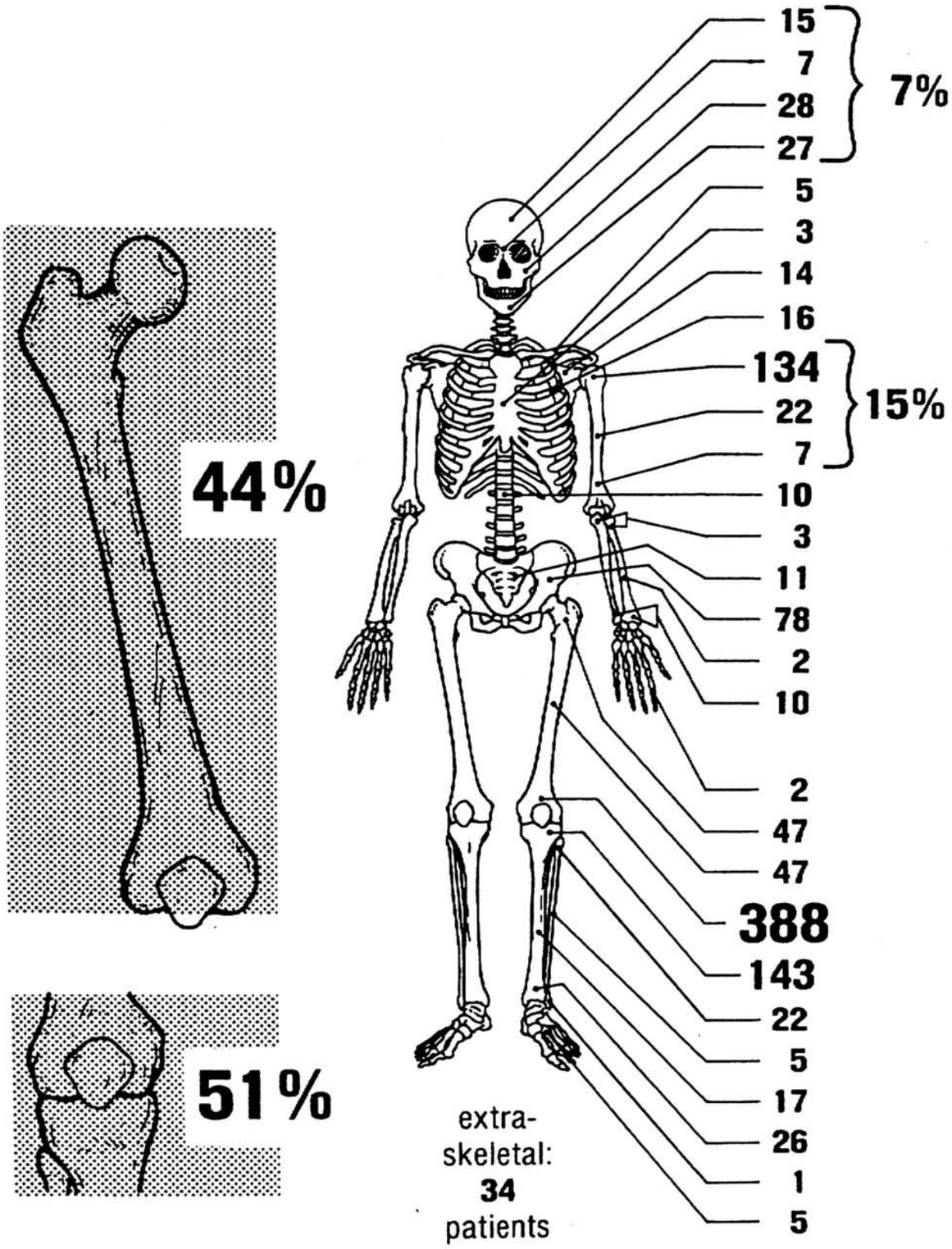


Figure 1.3 Skeleton distribution in 1095 patients with osteosarcoma diagnosed and treated at Memorial Sloan-Kettering Cancer Center 1921 through 1979 (Huvos, 1991).

(Table 1.1). This system is based on the interrelationship of three factors: Grade (G), Site (T) and Metastasis (M). This system serves as a useful guide in the selection of an appropriate treatment and definitive surgical procedure (Enneking, 1986; Kerns & Simon, 1983; Wolf & Enneking, 1996).

1.1.4.1Grade

The Grade is an assessment of the biologic aggressiveness of the lesion. It combines the histological assessment, radiographic assessment and the clinical aspect. The three stratification of grade are G_0 (Benign), G_1 (Low-grade malignant) and G_2 (High-grade malignant).

1.1.4.2Site

There are three strata of the anatomical setting : T_0, T_1 and T_2 . Both T_0 and T_1 are intracompartmental. For T_0 , the lesion remains confined within the intact capsule while for T_1 , the lesion has extracapsular extension but all the lesions are within an anatomic compartment bounded by the natural barriers to tumor extension (Intracompartmental). In T_2 , lesion extends beyond compartmental barriers into the loosely bounded fasical planes and space that have no longitudinal boundaries (extracompartmental).

1.1.4.3Metastasis

There are only two strata of metastasis : M_0 and M_1. M_0 indicates no dence of regional or distant metastasis, whereas M_1 signifies either regional or distant metastasis.

Malignant lesions that are designated by Roman numerals : I, II, II are synonymy with low-grade, high-grade and metastasis. These three stages of sarcoma are further stratified into A and B depending on whether the lesion is anatomically intracompartmental (A) or extracompartmental (B). The characteristics of these malignant lesions are shown in Table 1.1. In osteosarcoma, most of the lesions (> 90%) occurred in stage IIB.

	I_A	I_B	II_A	II_B	III_A	III_B
Grade	G_1	G_1	G_2	G_2	G_{1-2}	G_{1-2}
Site	T_1	T_2	T_1	T_2	T_1	T_2
Metastasis	M_0	M_0	M_0	M_0	M_1	M_1
Clinical Course	Symptomatic indolent growth		Symptomic rapid growth	Symptomic rapid growth fixed mass pathological fracture	Systemic symptoms palpable nodes pulmonary symptoms	
Isotope scan	Increased uptake		Increased uptake beyond radiographic limits		Pulmonary lesions no increased uptake	
Radiography grade	II		III		III	
Angiogram	Modest neovascular reaction, involvement of neurovascular bundle		Marked nevascular reaction : no involvement of neurovascular bundle	Marked nevascular reaction : no involvement of neurovascular bundle	Hypervascular lymph nodes	
CT	Irregular or brosched capsule but intracom-partmental	Extracompart-mental extension or location	Broached capsule : intracompart-mental	Broached capsule : extracompart-mental	Pulmonary lesions or enlarge nodes	

Table 1.1. Stages of Malignant Muscoskeletal Lesions (Enneking,1986)

1.1.5 Histological Features

Osteosarcoma can be divided into histologic subtypes of fibroblastic, chondroblastic, osteoblastic, telangiectatic and mixed, on the basis of the predominant cell formation or the pattern of growth in the lesion. Different subtypes of osteosarcoma reveal a significant degree of expression in alkaline phosphatase. The chondrosarcoma and the fibrosarcoma can be separated from the chondroblastic and fibroblastic osteosarcoma by the absence of alkaline phosphatase immunohistostaining (Table 1.2).

1.1.6 Clinical Features

The most common clinical symptoms of osteosarcoma is pain at tumor site, often irradiating to the neighboring joint, which begin insidiously and intermittently. The pain then progresses to severe, constant pain and is not relieved by rest, immobilization or ordinary analgesics. Swelling of the affected part and limitation of joint motion were later developed. The symptoms will be more predominant if there is a pathological fracture. However, pathological fracture is not common and is usually found in the predominantly osteolytic lesions.

Local inflammatory signs and venous stasis often occur in advanced lesions. The duration of symptoms prior to diagnosis is extremely variable, averaging not more than a few months.

Most of the patients draw their attention to their pre-existing bone lesion through their injuries. Ewing (1935) referred to this occurrence as "traumatic determination", i.e., trauma reveals more malignant growths than it products.

Approximately 10-20% of the patients presents with clinically evident metastasis at diagnosis, most commonly in the lungs (Marina *et. al.*, 1992). The prognosis continues to be poor in these patients.

		ALP expression
Osteosarcoma	Osteoblastic	+++
	Chrondroblastic	+++
	Fibroblastic	++
	Telangiectatic	++
Chondrosarcoma		-
Fibrosarcoma		-

Table 1.2. Immunohistochemical finding in different subtypes of osteosarcoma in comparison with other chondroid tissue.

1.1.7 Radiological Features

The first diagnostic test for a patient with suspected malignant bone tumor is a plain radiograph. Radiographs of osteosarcoma may show lytic or mixed lytic and destructive lesions, sometimes with calcification evidence of new bone formation.

Most of the osteosarcoma arise centrally, destroy the cortex bone and invade the surrounding soft tissue, resulting the classic reactions exhibited by the periosteum. The most common radiolofical features are produced after these processes. The earliest recognizable radiographic change includes minimal irregular periosteal new bone formation in the metaphyeal, with the underlying bone exhibiting a localized motterled radiolucent or radiopaque area and periosteal reaction may appear as a fine "sunburst" or a single vertical periosteal layer (Figure 1.4).

In advanced stage, definite cortical destruction and breakthrough can be seen clearly. Moreover, there is obvious periosteal elevation, so called "Codman's triangle", and spicule formation (Figure 1.5). Codman's triangle is a manifestation of extreme periosteal elevation forming an acute angle with the cortex. However, the periosteal new bone formation is just a reactive response to the lifting of the periosteum and is not specific for osteosarcoma since it may be seen in Ewing's tumor as well.

Other than plain radiography, modern imaging technique, such as computed tomography (CT) and the magnetic resonance imaging (MRI), also play important role in diagnosis and monitoring of the osteosarcoma. CT examination is mainly applied in appendicular and axial skeleton. It is helpful for guidance of the biopsy and to establish the intramedullary extent of the tumor and demonstrate the presence of skip metastasis (Hubbard, 1983). Magnetic resonance imaging accurately assesses tumor size and the extent of intramedullary tumor involvement.

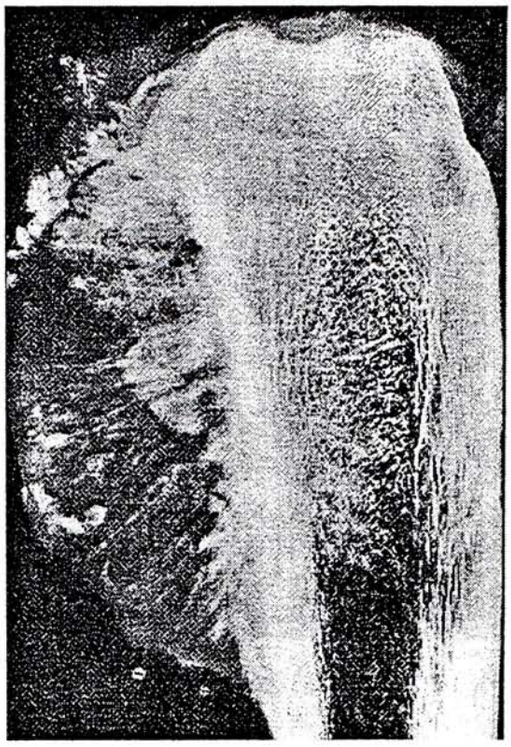


Figure 1.4 Male/22, osteosarcoma of upper tibial end, X-ray radiography showing "sunbrust" peristeal reaction (Schajowicz, 1994).

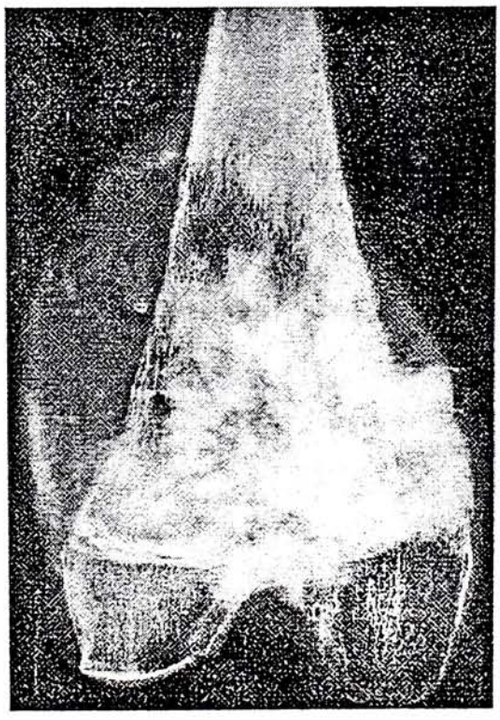


Figure 1.5. Male/13, Osteosarcoma of lower metaphysis of femur, X-ray radiograhy showing foramtion of typical Codman's triangle (Schajowicz, 1994).

1.1.8 Molecular genetics

Most osteosarcoma harbor complex and multiple genetic aberrations, with characteristically bizarre karotypes (Biegel et. al., 1989). Patients with heritable retinoblastoma who bear constitutional mutations or losses at the retinoblastoma gene locus have a much higher chance of osteosarcoma. Loss of heterozygosity at the retinoblastoma gene locus on chromosome 13q and allelic loss or mutation in p53 gene in the short arm of chromosome 17 is a common finding in patients with osteosarcoma (Wadayama *et al.*, 1994). Allelic losses on the long arms of chromosome 3 and 18 and amplifications of the SAS-MDM2 locus on chromosome 12q are frequently found in osteosarcoma too (Tarkkanen *et. al.*, 1995). The retinoblastoma and p53 gene products, known as tumor suppressers, are important regulators of cell cycle, whereas MDM2 gene product interacts with p53 in cell cycle regulation.

1.1.9 Treatment

Prior to 1970, complete surgical excision was the only treatment option available to achieve tumor control prior to the development of adequate radiation therapy and chemotherapy. Unfortunately, even with the complete resection of the tumor, local (95%) and systemic recurrence rates (80 - 90%) were very high (Campanacci & Laus, 1980; McKenna *et al.*, 1966) and cure rate is low, varying from 5 to 17% (Marcove *et. al.*, 1970).

Adjuvant chemotherapy for osteosarcoma was initiated in the early 1970s. The results from these early adjuvant chemotherapy trials were very encouraging. Adjuvant chemotherapy consists of using anti-neoplastic drugs after local control of the tumor by surgery or radiation. It decreases metastases by eliminating mircometastic tumor deposits that may be present in the lungs, bone, bone marrow, lymph nodes and other sites. Moreover, the primary tumor size is reduced so that the subsequent surgery or radiation treatment for local control is technically easier, allowing limb salvage

(preservation of the extremity) and allograft reconstruction possible (Eilber *et. al.*, 1984).

With the current regime of combine adjuvant chemotherapy and surgical treatment in osteosarcoma, the diseases-free survival for osteosarcoma has been improved dramatically compared with the historical controls. Approximately 60% to 65% of patients with non-metastasis osteosarcoma of the extremity survive without evidence of recurrence (Velez-Yanguas & Warrier, 1996). The majority of current regimens incorporate adriamycin, cisplatin, and high-dose methotrexate (HDMTX).

With the improvement of both the survival and the quality of osteosarcoma patients, the need for a sensitive biochemical marker is raised. A sensitive biochemical marker allows us to identify patients with poor prognosis, monitoring the patients' response to treatment and detect early local recurrence or metastasis of the tumor.

1.2 Biochemical Markers of Osteosarcoma

1.2.1 Tumor Marker

Tumor marker is broadly defined to include any tumor-associated cell surface antigen or intracellular protein. They are often refers to substances in blood or body fluids of patients with cancer (Bates & Longo, 1987).

For an ideal biochemical marker in cancer, the marker should be produced solely by the tumor cells and be readily detectable in body fluids. It should not be present in healthy individual or patients with benign disease. The quantity of tumor marker should directly reflect the bulk of malignancy and correlate with the results of anticancer therapy. It should be present frequently enough and early enough in the development of a malignancy to be useful for screening. No marker described to date meets all of these criteria, but a marker will still have its clinical values if it can perform certain functions such as following the course of therapy and detecting recurrence.

To examine the clinical value of a biochemical marker, several questions should be answered. Concerning the measurement itself, what method of measurement can be used? What is the sensitivity and specificity of the assay? Will a cross-reacting substance give false positive results and what is the lower limitation of the assay?

In addition, the incidence rate of elevation of the marker should be high and the increase in the level of marker should relate to the stage of cancer and the prognosis of disease. The level of marker should also tell the physician whether the therapy has been effective and the normalization of the marker should indicate that all malignant disease has been eliminated.

1.2.2 Biochemical markers of bone turnover

The most commonly employed biochemical markers in bone activity are listed in Table 1.3. It consist of substances or molecules released into the circulation during the formation or resorption of bone.

For bone formation markers, serum osteocalcin is associated with the initiation of mineralization. It is the most abundant non-collagenous protein in the mineralized bone matrix and it is involved in the mineralization process of the bone (Akesson, 1995). Procollagen I C-terminal extension peptide (PICP) is released from the procollagen during the synthesis of type I collagen. Since collagen synthesis precedes mineralization, the amount of PILC molecule reflects the amount of mineralized bone (Risteli & Risteli, 1992).

Hydroxyproline, pyridinoline and telopeptides are the degradation product of collagen during the bone resorption and liberated into the circulation. They are all removed from kidney and thus their level are greatly affected by the kidney function (Epstein, 1988).

Of all biochemical markers in bone turnover, serum alkaline phosphatase (ALP) is the most widely used. ALP was clustered at the surface of the osteoblasts and is responsible for the mineralization process (Anderson, 1989).

Bone Formation	Bone Resorption
Serum / Plasma Alkaline Phosphatase	Urine Hydroxyproline
Serum Osteocalcin	Urine Pyridinoline
Serum Procollagen I C-terminal Extension Peptide	

Table 1.3. Biochemical markers of bone turnover.

1.2.3 Change of biochemical marker in osteosarcoma

Davies *et al* (1991) compared the effectiveness of several biochemical markers of bone turnover in monitoring of osteosarcoma. These markers included serum ALP level, serum osteocalcin and urinary hydroxylproline. By measuring these biochemical markers in a 65-year-old woman with osteosarcoma in right knee, they found that serum osteocalcin and hydroxylproline did not show any response to the onset of osteosarcoma. Whereas serum ALP increased rapidly during relapse and returned to low level after the resection of tumor (Figure 1.6). This suggested that serum alkaline phosphatase should be a better choice as a biochemical marker of osteosarcoma.

1.3 Alkaline Phosphatase (ALP)

Alkaline phosphatase (EC 3.1.3.1) is a trivial name of a family of isozymes with similar catalytic properties that hydrolyze a large variety of phosphomonoesters at a rather high pH, usually pH 8 to 10.7 [orthophosphoric-monoester phosphohydrolase (alkaline optimum)]. This enzyme is widely distributed in nature and is found in large range of organisms from plant, algae to vertebrates.

ALP is a metalloenzyme with zinc being an integral part of the molecule. The native enzyme has four zinc atoms, two responsible for the catalytic activity while the other two are for the structural integrity. Other than zinc, serine is also presents in the active site and take part in the catalytic reaction. In addition, inorganic phosphate is also an integral part of the enzyme (McComb *et. al.*, 1979). The 3-dimensional structure of the ALP is illustrated in Figure 1.7.

Most ALP exists in dimer form with molecular weight ranging from 70,000 to 180,000. Moreover, many mammalian enzymes contain relatively large amounts of carbohydrates, especially sialic acid. Eukaryotic ALP are membrane-bound enzymes anchored to the exterior of the cytoplasmic

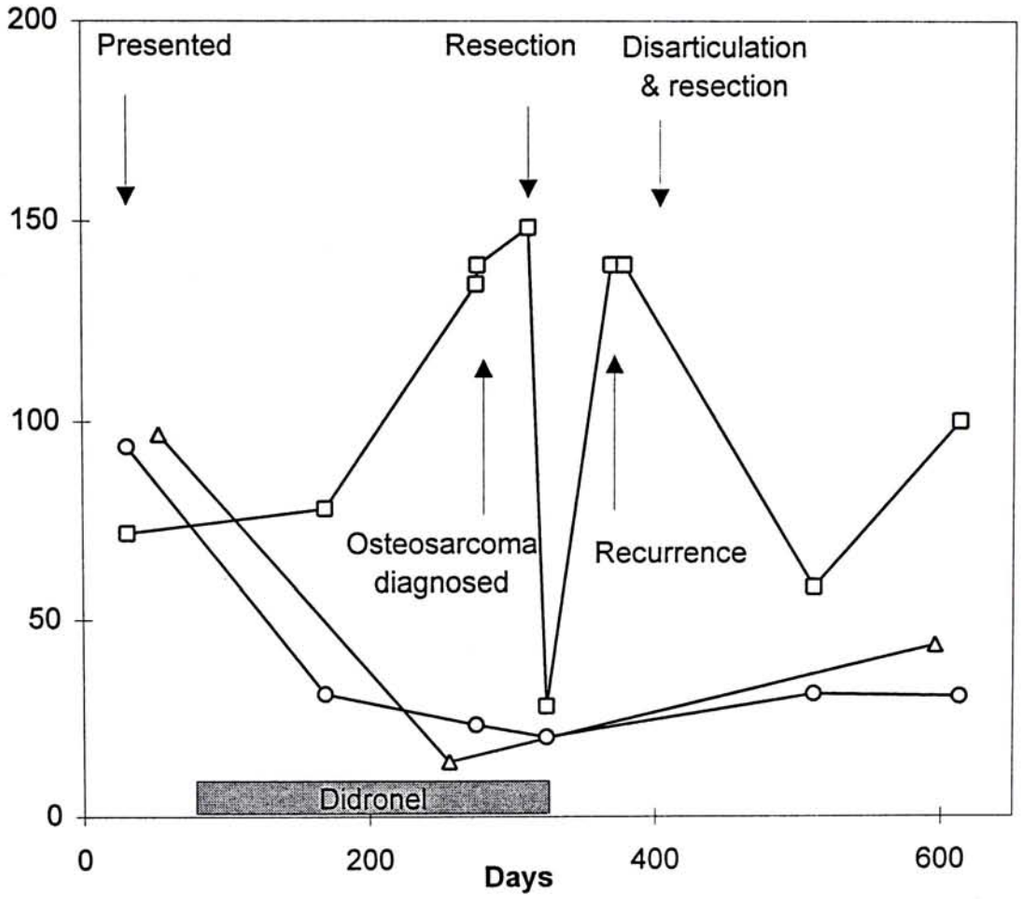


Figure 1.6. Relationships among serum alkaline phosphatase, serum osteocalcin and urinary hydroxyproline excretion during the treatment and progression of osteosarcoma. (Davies, 1991)
–□–□– Alkaline phosphatase (U/L); -O-O- osteocalcin (ng/ml);
–Δ–Δ– hydroxyproline excretion (umol/day)

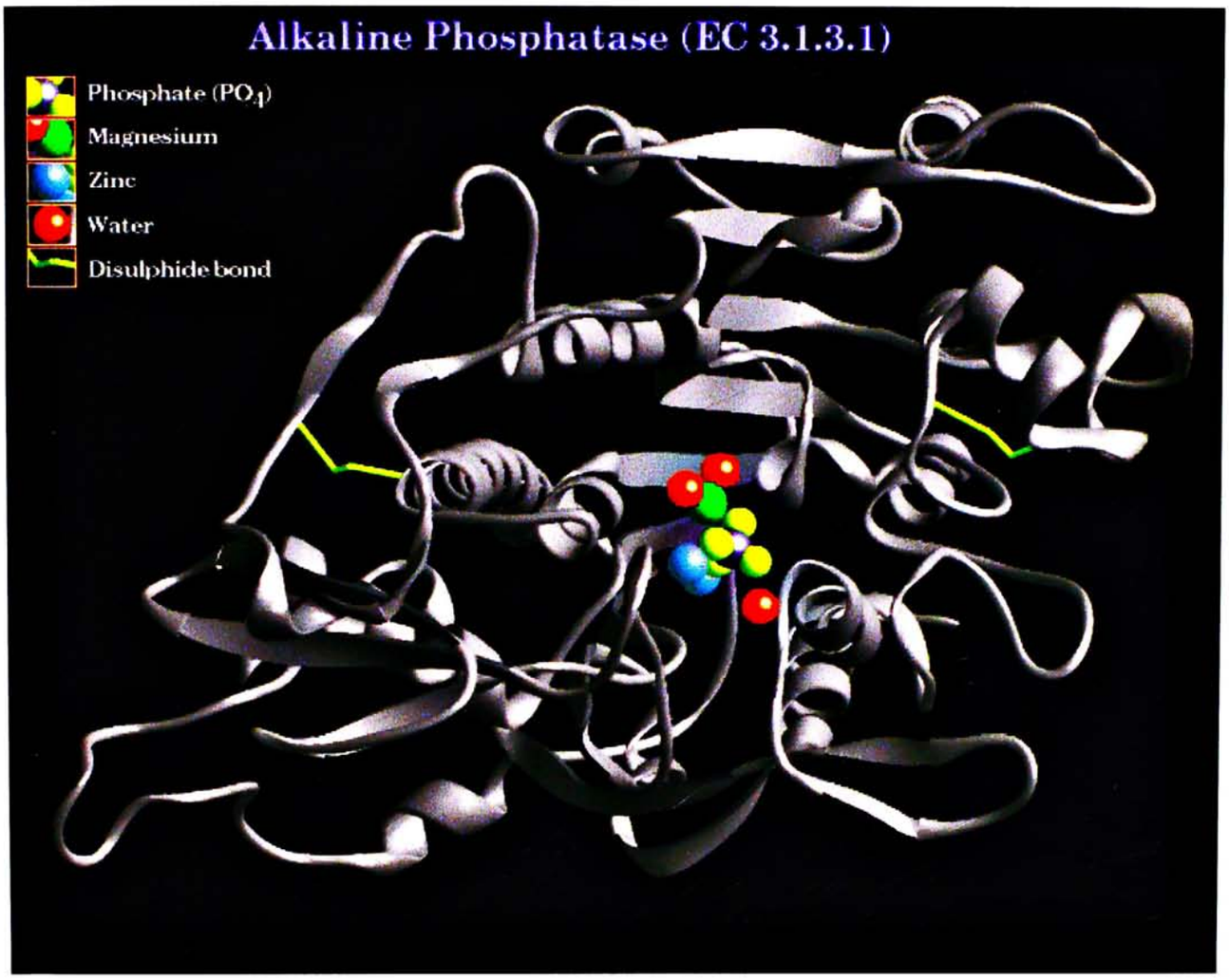


Figure 1.7 Three dimensional structure of Alkaline Phosphatase of *Escherichia coli*. (From internet)

membrane via a novel complex structure identified as the glycosyl phosphatidylinositol (GPI) anchor (Howard *et. al.*, 1987; Wong & Low, 1992; McComb *et al.*, 1979).

1.3.1 ALPs Family

In mammals, ALP consists a group of true isozyme encoded for at least four different gene loci: The L/B/K locus determines the so-called liver/bone/kidney of "tissue specific" ALP and is expressed in vitally all tissues. This L/B/K gene is located at the end of the short arm of chromosome 1, bands p36.1-p34 (Weiss *et. al.*, 1988). The other three are the tissue specific ALP genes, i.e. intestinal ALP (IALP), placental ALP (PALP) and germ cell ALP (GCALP). They are clustered at the end of the long arm of chromosome 2, bands q34-37, while a single nonspecific ALP gene is located at the end of the short arm of chromosome 1, bands p36.1-p34 (Millan & Fishman, 1995). As their name stated, they are only expressed in the specific tissue or only in trace amount in other tissues. The whole family of the ALP isozymes was shown in Figure 1.8.

The close association of these three loci of the tissue specific ALP presumably reflects their common, comparatively recent, evolutionary ancestry. The three tissue specific ALP genes and their products have correspondingly similar sequences of bases and amino acids; these similarities are particularly marked in the placental and germ cell ALP with 98% protein homology. The homology between the intestinal and placental ALP is 86.5% but only 56.6% to the tissue nonspecific ALP (Moss, 1992). The enzymes are synthesized as propeptides varying in length from 524 in L/B/K ALP to 535 in the placental and germ-cell ALP (Millan, 1988).

The reasons for the characteristic differences in the expression of the various loci in different tissues, and indeed in different cells within the same tissue, are not known. In the purest sense of the term, intestinal, placental and tissue-nonspecific ALPs is true isozymes because their amino acid sequence

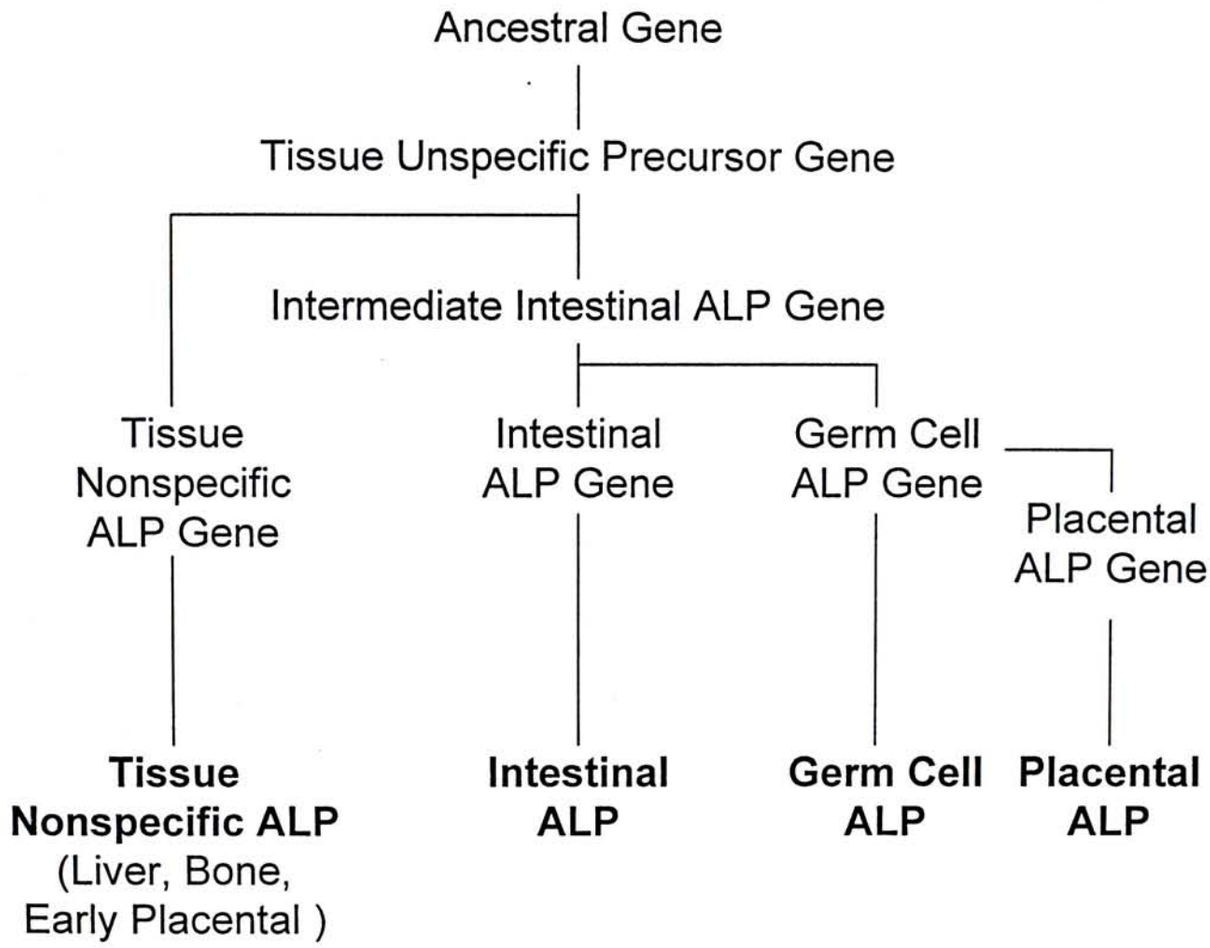


Figure 1.8. Evolution of the ALP gene family (Fishman, 1990)

are different. For the tissue-nonspecific ALP isoforms, such as liver- and bone-specific ALP, difference arises from the post-translational modifications of the same gene product. Thus strictly speaking, they are considered as isoform but not isozyme.

The amino acid sequences of the L/K/B isozymes are highly homologous, displaying 90% to 98% identity. On the other hand, the tissue specific ALP is only 50% to 60% homologous with any each other (Millan & Fishman, 1995).

Much less is known about the structures of the carbohydrate moieties of these glycoproteins. Differential glycosylation of the tissue-nonspecific ALP gives rise to tissue-specific isoform. These sugar moieties are believed to protect the enzyme from the rapid removal from the circulation through binding by the asialoglycoprotein receptors of the liver (Komoda & Sakagishi, 1978). It is known that the carbohydrate side chain of IALP is not terminated by sialic acid. Moreover, the carbohydrate moieties of liver-specific ALP (LALP) and bone-specific ALP (BALP) may depend on the way sialic acid and the O-linked sugar chain are linked to the molecules (Miura *et. al.,* 1994).

1.3.2 Membrane Binding

ALP is anchored to the membrane by a phosphatidylinositol glycan (GPI) which is covalently-link to the carboxyl-terminus of the peptide chain (Figure 1.9). The two fatty acyl residues of a molecule of phosphatidic acid are inserted into the outer layer of the membrane where they are non-covalently anchored by hydrophobic interactions with the lipid components of the membrane (Low & Zilversmit, 1980).

ALP is released from the tissues into the serum in a variety of pathological and physiological situations. Several mechanisms of release of GPI-anchored ALP molecules can be envisaged. ALP is nonspecifically released by detergent-mediated solubilization, in which the ALP released from the membranes with its GPI-anchor intact. This form of release is able to

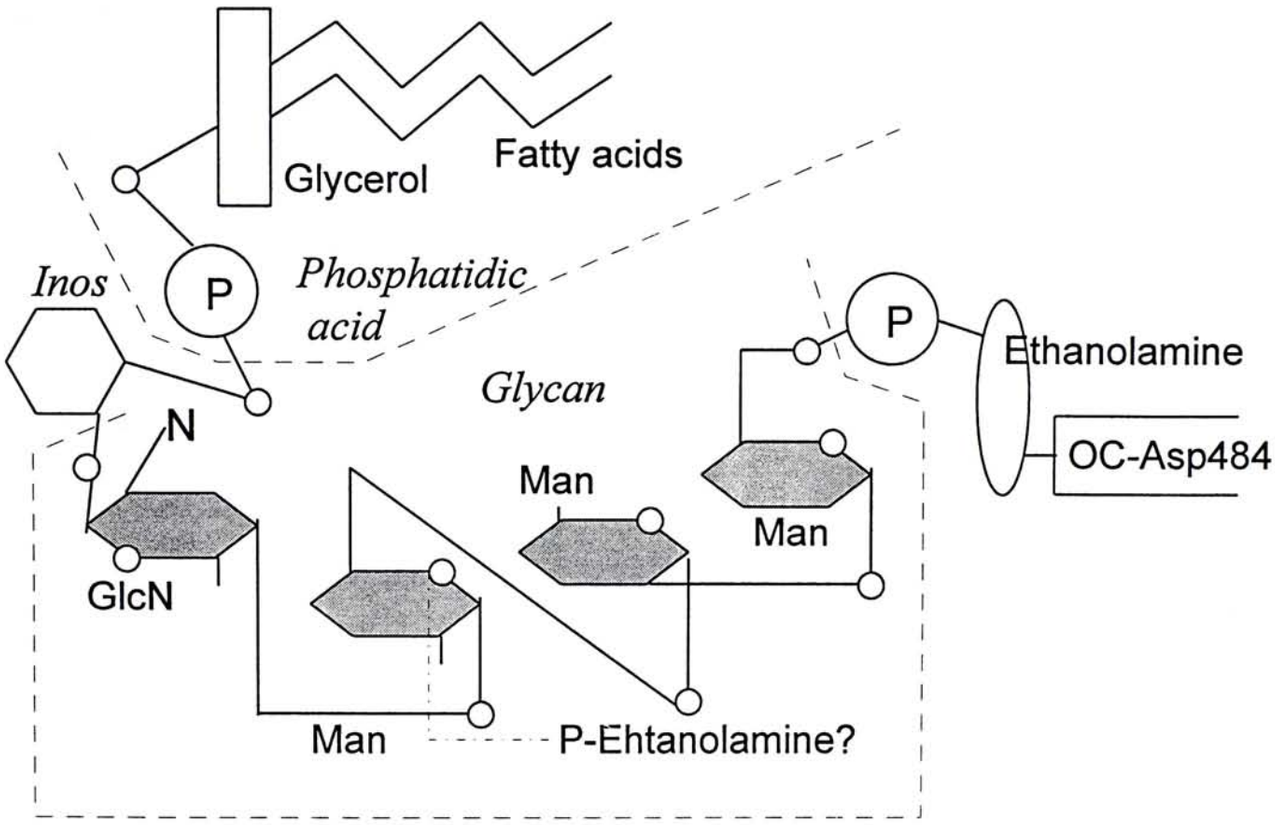


Figure 1.9 Structure of the glycan phosphatidylinositol membrane anchoring domain of human placental ALP (Moss, 1994).

P = phosphate, Man = mannose, GlcN = glucosamine, Inos = inositol

undergo post-release aggregated, either with themselves or with other hydrophobic molecules, result in a high Mr isoform of ALP. For example, bile provides a favorable environment for ALP-lipid complexion and the ALP in bile is predominantly present as high M_r isoform (Moss, 1994; Raymond *et. al.*, 1991).

Another means of ALP release is by specific enzyme mediated cleavage of covalent bond of the GPI anchor. The GPI linkage of ALP is susceptible to cleavage by two phosphatidylinositol-specific phospholipases C and D (PI-PLC of PI-PLD) (Figure 1.10). Phospholipase can promote the solubilization of ectoenzyme from cell in vitro and releasing the hydrophilic form of ALP. PIPLC appears to be principally an intracellular enzyme and active PIPLD is present in human plasma (Davitz *et. al.*, 1987) and it is this enzyme that is thought to be involved directly or indirectly in the solubilization of GPI-linked proteins from cell membrane.

Since proteolytic cleavage is unlikely to contribute directly to the solubilization of ectoenzyme from cell surfaces, it is believed ALP released in serum through the following steps: ALP is solubilized from the biliary and plasma surfaces of hepatocytes by detergent action or membrane turnover. PIPLC in plasma removes phosphatidic acid and renders the ALP more hydrophilic low M_r form.

1.3.3 Biochemical Function and Physiological Role of ALP

The *in vivo* functions of ALP are believed to be (Price, 1993):

1. Phosphohydrolysis of organ phosphomonoesters of low molecular mass;
2. Phosphotransferase activity;
3. Protein phosphatase activity.

ALP has little preference for a particular substrate and will hydrolyze all phosphomonoester. Hydrolysis of phosphomonoester proceeds via cleavage of O-P binds with an activation energy of 6000 ~ 10,000 cal/mol following first order and zero-order kinetics. Catalysis includes

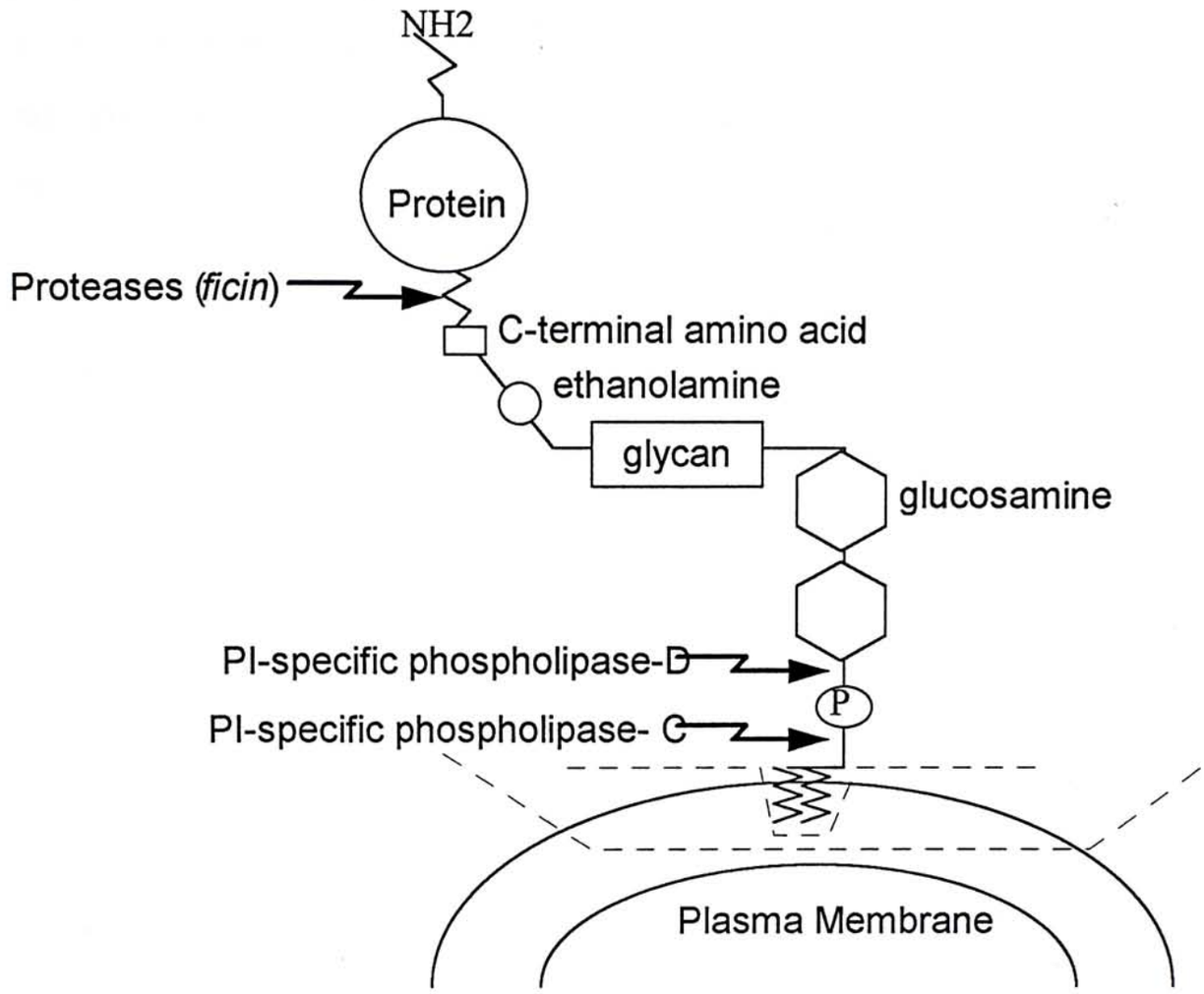


Figure 1.10. Schematic representation of the anchoring of ALP (monomer is shown) in the plasma mambrane (Moss, 1994). Sites of cleavage by protease, PIPLC and PIPLD (arrows) and solubilization of mambrane (dotted line) are indicated.

phophorylation of a serine residue at the active site, followed by transfer of the phosphoryl group to either water (phosphohydolysis) or an organic acceptor-alcohol (phosphotransferase) with the transphosphorylation more preferred because of the hydrophobic nature of the plasma membrane-bound ALP (Stinson *et al.*, 1987).

The endogenous substrates of ALP are believed to be: phosphoethanolamine (PEA), inorganic pyrophosphate (PPi) and pyridoxal 5'-phosphate (PLP) (Russell, 1965; Whyte *et al.*, 1995). The widely distribution of ALP in nature suggested that this enzyme is involved in fundamental biochemical processes. The suggested physiology functions of ALP are listed below, with special description in bone-specific ALP.

Embryonic development and cell differentiation -- One of these fundamental processes may be embryonic development and cell differentiation, in which ALP guide the migration of the embryonic cell. It is believed that the GCALP interact with the extracellular matrix proteins and therefore serve as the cell guidance molecule during migration of germ cells down the gential ridge.

Regulation of lipid transport -- Intestinal ALP may be involved in lipid transport in the intestine, with the absorption of the lipid which bind to the intestinal ALP.

Regulation of renal phosphate transport -- ALP is indirectly involved in the transport of the Pi, evidenced by the decrease of Pi reabsorbance in kidney after the administration of the ALP inhibitors (Petitclerc & Plante, 1981). Moreover, ALP activity is highest in the early proximal tubule in kidney where Pi transport is the highest.

Transport of IgG molecule during pregnancy -- PALP permits the transmission of functional antibodies to the fetus through the placenta by acting as a Fc receptor (Makiya *et. al.*, 1982).

Regulation of pancreatic chloride channels -- Membrane bound ALP plays an important role in the regulation of a phosphorylated low-conductance chloride channel in human pancreatic duct cells (Becq *et al.*, 1993).

Metabolism of PLP in Brain Microvessels -- ALP is a key enzyme of the blood brain barrier regulated by insulin. ALP may have a role in the metabolism of PLP, a cofactor of metabolic enzymes such as glutamate decarboxylase and glutamate transminase in the neural system. Brain ALP can significantly be inhibited by insulin (Catalan *et. al.*, 1988).

ALP in Liver -- Although half of the circulating ALP in serum come from liver, no precise function of the LALP can be attributed since gross deficiency of liver ALP in congenital hypophosphatasia does not give rise to any obvious clinical manifestations.

Bone formation

The bone-specific ALP has an important role in skeletal mineralization (Anderson, 1989). BALP appears as tetrameric protein localized in the plasma membrane of the osteoblasts. Its activity is as 20 times higher in bone matrix vesicles, functionally active shed plasma membrane fragment of the osteoblast and the initial site of hydroxyapatite crystal formation. Robison (1923) first recognized the role of ALP in skeletal mineralization.

BALP takes part in the bone mineralization processes by hydrolysing the organic phosphate esters resulting in local high Pi concentration which facilitates the precipitation of calcium phosphate. Moreover, ALP can destroy the physiological crystal growth inhibitors such as inorganic pyrophosphate and ATP through its hydrolase activity. In addition, it acts as Pi transportor and actively transports Ca^{2+} or Pi through its ATPase activity (Whyte, 1994).

Moreover, the extracellular matrix binding domain of BALP may be important in directing the migration of matrix vesicles along collagen fibers during the process of bone mineralization.

1.4 Normal Values of serum ALP

In a health normal subject, the serum ALP level is mainly contributed by the bone and liver ALP isoform. The activity of both isoforms is markedly dependent on age (Fleischer *et. al.*, 1977; Leung, *et. al.*, 1993). The effect of bone growth, leading to an increase in BALP, and therefore to serum ALP, in children and adolescents is well established. After the growth spur, serum ALP drops dramatically, again due to the drops of BALP (Moss, 1982). On the contrary, LALP activity in serum increases steadily throughout life, and there is also some increases in BALP in the elderly (Figure 1.11).

IALP activity rises following the ingestion of a fatty meal. The presence of intestinal ALP is more frequent in the serum of healthy individuals with blood groups B or O who are secretors (Langman *et al.*, 1966). As previously stated, placental ALP plays an important role in the pregnancy, it is therefore no surprise to see that PALP appears in serum of the pregnant mother. PALP is observable in maternal circulation after 5 to 6 weeks of conception (Okamoto *et al.*, 1990). Placental ALP is also present in trace amounts in serum from healthy, non-pregnant individuals (Hendrix *et. al.*, 1990).

1.5 Clinical Applications of ALP

The importance of the measurement of ALP activity in serum has not decreased during the half century since its introduction into diagnostic enzymology. Indeed, the growth of multiparameter biochemical profiling has consolidated its position amongst the most frequently performed tests in clinical chemistry. Main application of serum ALP level lies on the bone and hepatobiliary diseases (Moss, 1992).

ALP measurement was first applied to investigate bone diseases on a theoretical basis established by Robison (1932) and Kay (1929), through their demonstration of the association between an elevated ALP activity in serum and increased osteoblastic activity. Elevated serum ALP activity occurs in

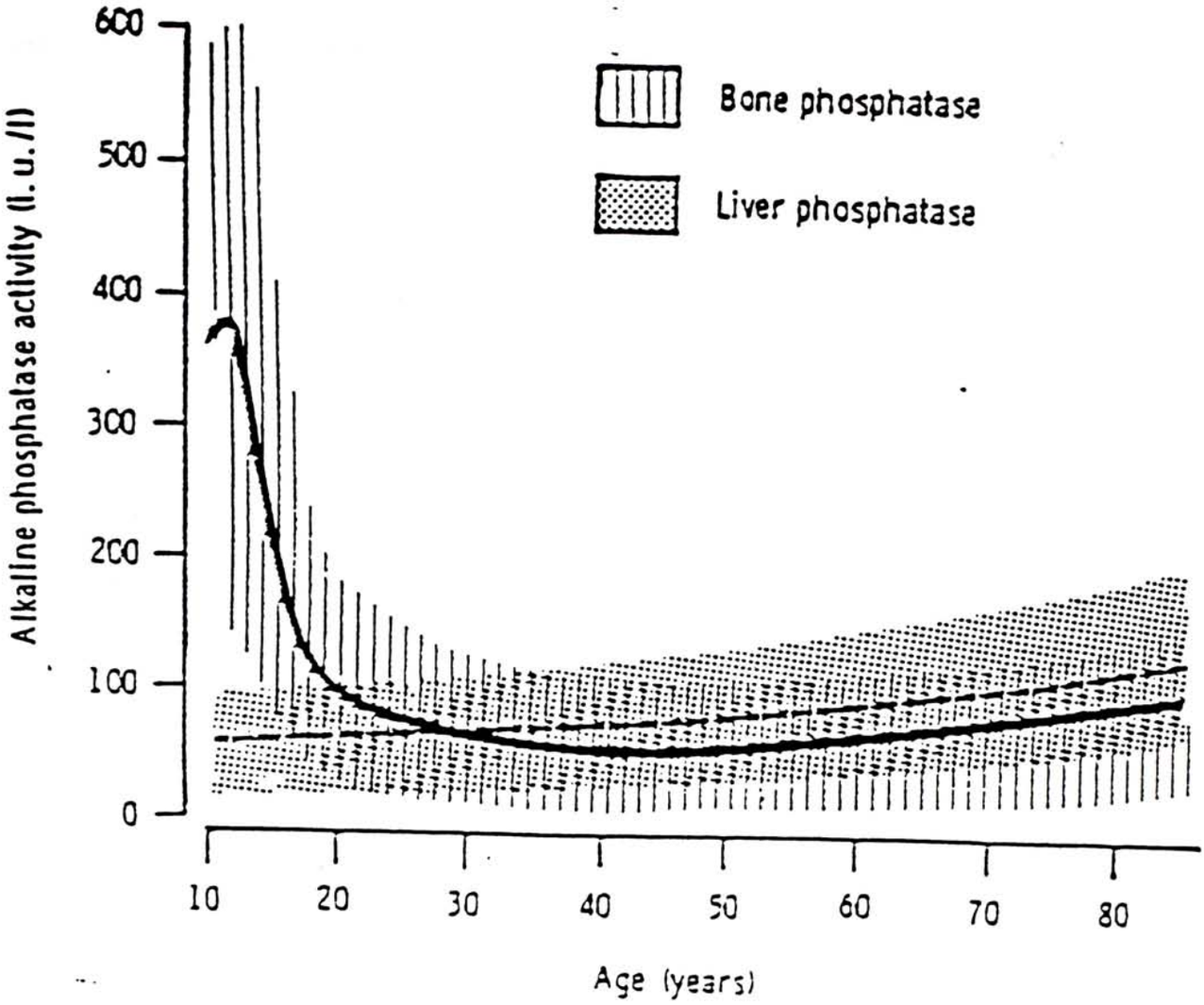


Figure 1.11. Change of the relative activities of bone and liver alkaline phosphatase in human serum with age. (Moss,D.W., 1982)

many skeletal disorders which reflects bone rebuilding processes increase in osteoblastic activity. This generalization remains the basis for the interpretation of serum ALP values in bone activity.

The highest serum ALP levels are generally encountered in Paget's disease of bone and in broad terms the increase reflects the extent of the disease. The increase can be up to 25 fold of the normal values (Joplin & Steveson, 1990). Elevated serum ALP levels are also present in patients with osteomalacia and rickets. Malignancy of bone, especially osteosarcoma, can lead to elevations of serum ALP level which will be discussed with more details in the later section. In addition, during the healing process of the fracture, serum ALP level is increased with an increase in the osteoblastic activity.

Later, the raise of serum level was proven also related to hepatobiliary disease (Roberts, 1930) in which the ALP levels are normal or only moderately elevated in hepatitis, but are markedly raised in obstructive jaundice. The large elevation of serum ALP is mainly caused by the LALP isoform. The obstruction to flow of bile results in regurgitation of bile constituents into the blood. Moreover, it also causes the *de novo* synthesis of ALP at the canailicular face of the hepatocyte and thus increasing the circulating ALP (Price, 1993). The ALP level always returns to normal after removal of the obstruction.

A wide range of clinical conditions may also give rise to an increase in the serum ALP activity, such as rheumatoid arthritis, ankylosing spondylitis, renal disease. Pregnant women will show increase in ALP levels. However, the main clinical interest centers on the elevation of serum ALP in patients with diseases involving the liver or bone. The main clinical questions are concerned with:

1. detecting the presence of liver or bone disease;
2. determining the origin of an unexpected elevation of serum ALP;
3. monitoring the changes in the activity of the disease.

Thus, the major requirement is for methods that provide accurate and precise quantitation of different ALP isoforms, especially the bone and liver ALP isoforms.

1.6 Separation, identification and quantification of ALP isozymes

There are a wide ranges of techniques available for the characterization and quantitation of the multiple forms of ALP in serum. The techniques depend on the physicochemical properties, the nature of the catalytic site or protein structure and post-translation modification of the different isoforms.

1.6.1 Thermostability

Semiquantitation fractionation of different ALP isozymes can be achieved according to their thermostability (Patitclerc, 1976). The placental and germ-cell ALPs are remarkably thermostable. They may be heated at 65°C for an hour or more without loss of activity (Neale *et. al.*, 1965). In contrast, the intestinal and the tissue-nonspecific ALP are rapidly inactivated under these conditions (Table 1.4) but the intestinal ALP is more thermostable than other tissue-nonspecific ALPs. LALP in serum is found to be slightly more thermostable than the BALP isoform (Whitby & Moss, 1975).

1.6.2 Inhibition studies

Various low molecular weight substrates show differential inhibition of the different ALPs (Harris, 1989). The effects of various kinds of inhibitors are listed in Table 1.5. All these inhibitors are stereospecific and non-competitive. The placental, germ-cell and intestinal ALPs are about 30 times more sensitive to inhibition with L-phenylalanine than the tissue non-specific ALP. On the other hand, the tissue non-specific ALP are very much sensitive to L-homoarginine and levamisole inhibition than the tissue specific ALP. L-leucine characteristically gives stronger inhibition with germ-cell ALP than with other ALPs and thus valuable for differentiating the placental and germ-cell ALP. In addition, L-phenylalanyl-glycyl-glycine gives sharp differential

Human ALP	56°C (min)*	65°C (min)*
Tissue Nonspecific ALP	7.4	1.0
Intestinal ALP	>60.0	6.5
Placental and germ-cell ALP	--	>60.0

Table 1.4. Relative thermostabilities of Human ALPs

* Time in minutes required to give 50% inactivation of different human ALPs at 56°C and 65°C.

Inhibitor	L/K/B ALP*	Intestinal ALP*	Placental ALP*	Germ Cell ALP*
Lphenylalaine	31	0.8	1.1	0.8
L-Homoarginine	2.7	40	>50	36
L-Phenylalanyglycylglycine	30.6	3.7	0.1	2.9
L-leucine	13.1	3.6	5.7	0.6
Levamisole	0.03	6.8	1.7	2.7

Table 1.5 Effects of various inhibitors on different Human ALPs

* Concentrations (mM) of various inhibitors required to produce 50% inhibition of different human ALPs under standardized conditions.

inhibition between placental, intestinal and tissue-nonspecific ALP. This property also aids the differentiation between the placental and germ-cell ALP as inhibition pattern of germ cell ALP is more similar to the intestinal ALP.

1.6.3 Electrophoresis

The most common approach for identification of ALPs depends on electrophoretic separation, followed by staining for enzymatic activity (usually with α-naphthyl phosphatase as substrate and with diazonium salt to detect the liberated α-naphthol). However, zonal electrophorsis alone, no matter on which support media, fail to produce a distinct separation of the most important isoforms, namely liver and bone ALP isoform and allow accurate quantitation (Moss, 1982).

In case of paper (Baker & Pellegrino, 1954), starch (Jennings *et al.*, 1970) and cellulose acetate supports (Siede & Seiffert, 1977), the main liver isoform migrates as a compact band in the α_2 globulin region. The bone isoform is found to be immediately behind the liver bands and is characterized by more diffuse staining. Even with the molecular sieving supports such as agarose (Hagerstrand & Skude, 1976) and polyacrylamide (Walker & Pollard, 1971) support, the close proximity of the liver and bone fractions will still be seen.

The adult intestinal isozyme exhibits a slower mobility than the liver and bone ALP in the case of all supports. The placental ALP shows a more microheterogeneity in starch and polyacrylamide gel electrophoresis system (Robson & Harris, 1967) and appears as a discrete band overlaying the diffuse bone fraction.

The electrophoretic mobility of the bone isoform may be retarded by pre-incubation with neuraminidase (Moss & Edwards, 1984) or wheat germ lectin (Rosalki & Foo, 1984). This allows the quantitation of both the bone and liver fractions even when only small amounts of one component was

present. Both of these procedures depend on differences in the sialic acid residues of liver and bone ALP.

1.6.4 Isoelectric Focusing

It is possible to improve the resolution of electrophoretic techniques by employing isoelectric focusing (IEF). IEF is an electrophoretic technique in which amphoteric compounds are fractionated according to their isoelectric points (pIs) along a continuous pH gradient. Different ALP isozymes, since having different amino acid sequence, have different pI and thus can be separated from each others (Westermeier, 1993). For tissue nonspecific ALP isoform such as LALP and BALP, although the protein moieties are the same, the difference in the degree glycoslyation, especially the sialic acid content, will influence the net charge of the molecule, thus providing a means for separation.

By means of IEF technique, it was possible to separate ALP into at least 10 to 20 distinct bands (Griffiths & Black, 1987; Rosendahl *et. al.*, 1987). Unfortunately, the large number of bands appears to confuse. It is difficult to clarify the position of the clinically significant ALPs. However, it is useful for the characterization and identification of unusual ALP variants.

1.6.5 Affinity precipitation

Rosalki and Foo (1984) first reported that Wheat germ lectin (WGL) can preferably (80%) bind to the bone ALP isoform than the liver isoform and thus differentiating the bone fraction from liver fraction from serum. WGL binds to both N-acetylglucosamine and sialic acid residues and relative affinities for individual proteins will depend on the relative amount and accessibility of either or both of these sugars.

The same groups (Rosalki & Foo, 1986) later pointed out that the biliary fractions had to be dissociated by treatment with Triton X-100 to avoid the co-precipitation of this portion. Several commercial developed kits of BALP measurement (IsoALP, Boehringer Mannheim) are using this WGL

binding properties. However, some authors pointed out the reproducible of the assay is affected by quality of the WGL (Behr & Barnert, 1986; Desoize, *et. al.*, 1986).

1.6.6 Immunological studies

The first antibody against ALP was raised in 1954 with specificity towards dog intestinal ALP (Schlamowitz, 1956). The intestinal and placental ALP show some structural similarities and early polyclonal antisera demonstrated large cross reactivity (Lehmann, 1975). The achievement of specificity has only been successfully accomplished with the selection of monoclonal antibodies. Monoclonal antibodies against even different allelic forms of placental ALP can be achieved (Slaughter *et. al.*, 1983). Monoclonal antibodies for intestinal ALP without cross reactivity were also produced (Bailyes *et. al.*, 1988).

Several groups have attempted to differentiate between liver and bone isoforms using monoclonal antibodies but all antibodies raised show certain degree of cross reactivity due to the close structural similarity between the isoforms. Recent report of Hill and Wolfert (1989) demonstrates the production of monoclonal antibody against human bone ALP isoform with only 3% cross reactivity with the liver isoform, using BALP from human osteosarcoma cell line (Sa OS-2) as immunogen. Commercial ELISA kit (ALKPHASE-B®, Metra Biosystem) using monoclonal antibodies against BALP is now available for the direct capture and measurement of BALP in serum. The epitope of the monoclonal antibody recognizes the molecular conformation rather than the portion near sialic acid linkages in ALP molecules (Hata, *et. al.*, 1996).

1.7 Plasma BALP level as biochemical marker of Osteosarcoma

It is known that many patients with osteosarcoma have high serum total ALP levels. However, the prognostic significance of this finding is still controversial.

Bacci (1993) and his colleagues examined the serum ALP level of 656 patients with serum osteosarcoma and concluded that the pretreatment serum ALP levels have a prognostic value. His group demonstrated that the percentage of patients with increased serum ALP was significantly higher in the metastatic group than in the group with localized disease. Moreover, the relapse rate was significantly higher in patients with elevated ALP level than in normal level.

The reduction of elevated plasma ALP levels following the chemotherapy is a valuable guide to administration of chemotherapy. Rosen *et al.* (1974) demonstrated the effect of neoadjuvant chemotherapy on serum ALP values in a patient with proximal humeral osteogenic sarcoma. The decline of elevated values to normal levels signifies the sarcoma is responding favorably to chemotherapy.

Despite of the successfulness of these demonstrations, application of serum total ALP in bone diseases still lacks its specificity and sensitivity. As stated in the previous section, serum ALP is contributed not only by bone but also from liver origin, raise in serum ALP level is therefore not restricted in bone disease. As a cancer marker of bone tumor, elevated total serum ALP provides no indication about the site of metastases and its relationship to skeletal activity cannot be established without quantifying the amount of serum bone isozyme (Leung, *et al.*, 1993; Burlina, *et al.*, 1994).

It is therefore much more reasonable to use plasma BALP as a more specific indicator for osteosarcoma since it will avoid many complications which will lead to the raise of other forms of ALP, especially liver damage induced by the chemotherapeutic agents (Breithaupt & Kuenzlen, 1983). Moreover, BALP is a more sensitive biochemical marker of bone activity and its level truly reflects the bone activity (Liu *et al.*, 1996)

The application of plasma BALP measurement, in the past, is primary limited by the methods of measurement. Separation of plasma BALP from LALP can only be achieved by electrophoresis, which is too expensive and

cumbersome for routine measurement in clinical laboratory. Heat inactivation only allows semiquantitative measurement of plasma BALP. Accurate, easy to perform and less expensive methods for routine assay of plasma BALP level is only possible after the introduction of wheat germ lectin precipitation assay by Rosalki and Foo (1984). However, wheat germ lectin precipitation assay will yields falsely high values in patients with hepatobiliary diseases because of coprecipitation of the liver isoform (Withold & Rick, 1994) and BALP is not precipitated with similar efficiency in different sera (Farley *et al.*, 1993).

Recently, another accurate measurement of plasma BALP was introduced using the monoclonal antibody against human plasma BALP (Hata *et al.*, 1996). This ELISA (ALKPHASE-B®) captures the BALP in the plasma sample by the monoclonal antibody, coated in the bottom of the assay well, the BALP activity is then directly measured by using p-nitrophenyl phosphate as substrate.

1.8 ALP in malignancies

Numerous associations have been reported between the expression of tissue specific ALPs and malignancy (Herz, 1985). Tumors which express these ALP isozymes can be broadly divided into two groups: (1) those with an enhanced production of an isozyme normally expressed in the tissue (eutopic expression) and (2) those showing expression of one or more isozymes not identified in the normal tissue (ectopic expression). Moreover, many tumors show simultaneous expression of two or more different ALP isozymes (Nozawa *et al.*, 1990; Millan, 1992).

Placental ALP was one of the first enzyme recognized as an oncofetal protein by Fishman *et al.* (1968) in a patient (Regan) with disseminated lung cancer. It was termed Regan isozyme at that time and its functional characteristics were indistinguishable from those of the placental ALP biochemically, immunologically and structurally (Greene & Sussman, 1973).

The "Regan" isozyme has been reported in malignancies of lung, gastrointestinal tract, ovary, uterus, and in other tissues (Fishman *et. al.*, 1976). However, only some of the patients with these malignancies produce the enzyme.

Two years later, the Nagao isozyme was also found in cancer patients (Nakayama *et. al.*, 1970). This isozyme differs from PALP by its slower migration on starch gel and its sensitivity to leucine inhibition and it is most frequently expressed in germ cell tumor and ovarian cancer and actually serves as an useful tumor marker in those tumors. We now know that this Nagao isozyme expression are the results of eutopic expression of the Germ-cell ALP gene (Horfmann & Millan, 1993). Germ cell ALP is also ectopically expressed in tumors like pineal gland and thymus but only with low incidence of expression.

In 1972, Higashino and co-workers reported the presence of an intestinal-like ALP variant in patients with hepatocellular carcinoma and named this variant Kasahara isozyme after the first patient's name. By use of specific monoclonal antibodies, studies (Moss *et al.*, 1986; Imanishi *et al.*, 1989) shown that this isozyme is a heterodimer of PALP and IALP monomers which are similar to the fetal form of intestinal ALP. IALP appears to be primarily expressed in hepatomas although it has been found in other tumors as well.

Several different mechanisms underlying ALP expression in tumor cells can be envisioned (Millan & Fishman, 1995):

1. A functional involvement of ALP isozymes in tumorgensis;
2. ALP expression may represent an factor in a multifactorial etiology;
3. ALP expression may represent a close linkage of the ALP gene with a disease susceptibility gene;
4. ALP may be simultaneously deregulated with the disease susceptibility gene;

5. The expression of ALP isozyme genes could be the result of random chromosomal aberrations.

In osteosarcoma, eutopic expression of the bone-specific ALP is found. In 1993, Stinson and his group investigated the ALP gene expression in six human osteosarcoma cell lines in order to explore the possible molecular mechanisms for differences in specific activity of ALP. In six of the human osteosarcoma cell lines chosen, five of them express detectable levels of only the B/L/K ALP mRNA. However, in one of the cell lines which had the lowest expression of ALP mRNA (U-2 OS), they found a mixture of tissue-nonspecific ALP mRNA and at least one other form which indicated that an ectopic expression of ALP may be also present in osteosarcoma.

Aim of study

Increased level of serum alkaline phosphatase has long been used by other researchers as a biochemical marker in osteosarcoma. In the present project, we plan to test the possibility of using the bone-specific alkaline phosphatase as a more specific and sensitive plasma biochemical marker for diagnostic and prognostic purposes.

In the quantitative studies, plasma bone-specific ALP in osteosarcoma patients throughout the treatment periods were measured. Both wheat-germ lectin precipitation assay and the ALKPHASE-B immunoabsorption assay were employed for the measurements. The normal reference value of local Chinese population was determined. Comparison between the plasma BALP level of the patients at the time of diagnosis with the normal reference will provide useful information about the diagnostic and prognostic value of plasma BALP values. In addition, in order to evaluate the usefulness of plasma BALP level in monitoring the treatment progress, the change of plasma BALP level of the patients during the whole treatment plans up to the time of recurrence (if any) were followed.

In attempt to search for an even more specific biochemical marker for osteosarcoma, in the second part of the present project, we investigated the probable existence of the ectopic expression of ALP in osteosarcoma. Stinson (1993) reported the expression of ALP mRNA which is not originated from liver/kidney/bone in human osteosarcoma cell line U-2 OS. We explored the nature of this ectopic expression in this osteosarcoma cell line using the biochemical, isoelectric focusing and immunohistochemistry technique, and another human osteosarcoma cell lines (Sa OS-2) was employed as control. Furthermore, in order to screen for any ectopic expression of ALP in our clinical cases, we partially purified and identified the ALP expressed in

osteosarcoma patients' plasma samples and tumor tissues. In addition, immunohistochemistry using monoclonal antibody against human placental ALP were also applied to our osteosarcoma tissue paraffin section obtained.

Chapter Two: Materials and Methods

2. Methods and Materials

2.1 Plasma BALP measurement as a biochemical marker in Osteosarcoma

The plasma levels of BALP would be determined in normal Chinese and patients with primary osteosarcoma. These studies were in collaboration with the researchers of the Department of Orthopedics and Tramatology, the Chinese University of Hong Kong.

2.1.1 Patient groups

2.1.1.a Normal Subjects

A total of 137 normal subjects of local Chinese population (age 9-86) including 82 males and 55 females were recruited. These normal subjects have no past history or present signs and symptoms of liver or metabolic bone diseases. All subjects were divided into different age groups with group N1: age below 12; group N2: age between 12 to 16; and group N3: age above 16.

2.1.1.b Osteosarcoma Patients

A total of 49 patients (age 9 to 61) with 32 males and 17 females diagnosd as primary osteosarcoma were recruited for study. Consent from the patients (or patients' parents if he/she is below 16) were made. In general, patients received neo-adjuvant chemotherapy before operational remove of the tumor then follow by adjuvant chemotherapy. Serial blood samples of the patients were taken at admission, during the pre-operative chemotherapy, day before and after the operation, during the post-operative chemotherapy and at every follow-up clinic after the chemotherapy.

2.1.2 Collection and preparation of patient bloods samples of patients

Subjects' arm was placed in extend position. Cephalic or Median Cubital vein was located. The arm was tunaquited and the puncture site was sterilized with alcohol swap (BRAND). Venepunture was performed and 4 ml venous blood were aspirated with a 5 ml sterile syringe (TERUMO). The

tunaquit was unfastened and the syringe was withdrawn. The needle was removed and the blood was transferred immediately into a 4 ml heparinized tube with ammonium heparin coated on the wall of the tube (SARSTEDT). The tube was mixed gently for several times. Plasma sample was obtained by centrifugation at 1000 ×g for 10 minutes within 1 hour after blood collection. Plasma was aliquoted into 0.5 ml Eppenddorfs and stored at -70°C immediately.

2.1.3 Plasma Total ALP measurement

Measurement of the plasma TALP is based on a modification from the suggestions from the International Federation of Clinical Chemistry (IFCC) methods for Alkaline Phosphatase. (Tietz *et. al.*, 1983). The simplified equation for the reaction is shown in Figure 2.1. Appearance of the highly yellow colored products, p-nitrophenol, is monitored continuously at 405 nm at 25°C as the alkaline phosphatase in plasma catalyzed the hydrolysis of the colourless substrate, p-nitrophenylphosphate. The reaction takes places at a rigorously controlled pH 10.30 maintained by the transphosphorylating buffer, 2-amino-2-methyl-1-propanol. The rate of increase in yellow color is directly proportional to the ALP activity in the sample. One unit of activity is defined as that amount of enzyme which produce 1 μmole of p-nitrophenol per minute under the conditions of the assay procedure.

2.1.3.a Reagent

2-Amino-2-methyl-1-propanol (AMP) Buffer (0.5M, pH 10.3)

2-amino-2-methyl-1-propanol (Sigma) was warmed at 37°C water bath until it liquidified. 4.79 ml of this solution was added slowly in approximate 60 ml of deionized distilled water (ddH_2O). 0.04066g of $MgCl_2 \cdot 2H_2O$ (Sigma) was added to give a final concentration of 2 mM. The buffer is titrated with 1M HCl to pH10.3 and diluted to 100 ml with ddH_2O.

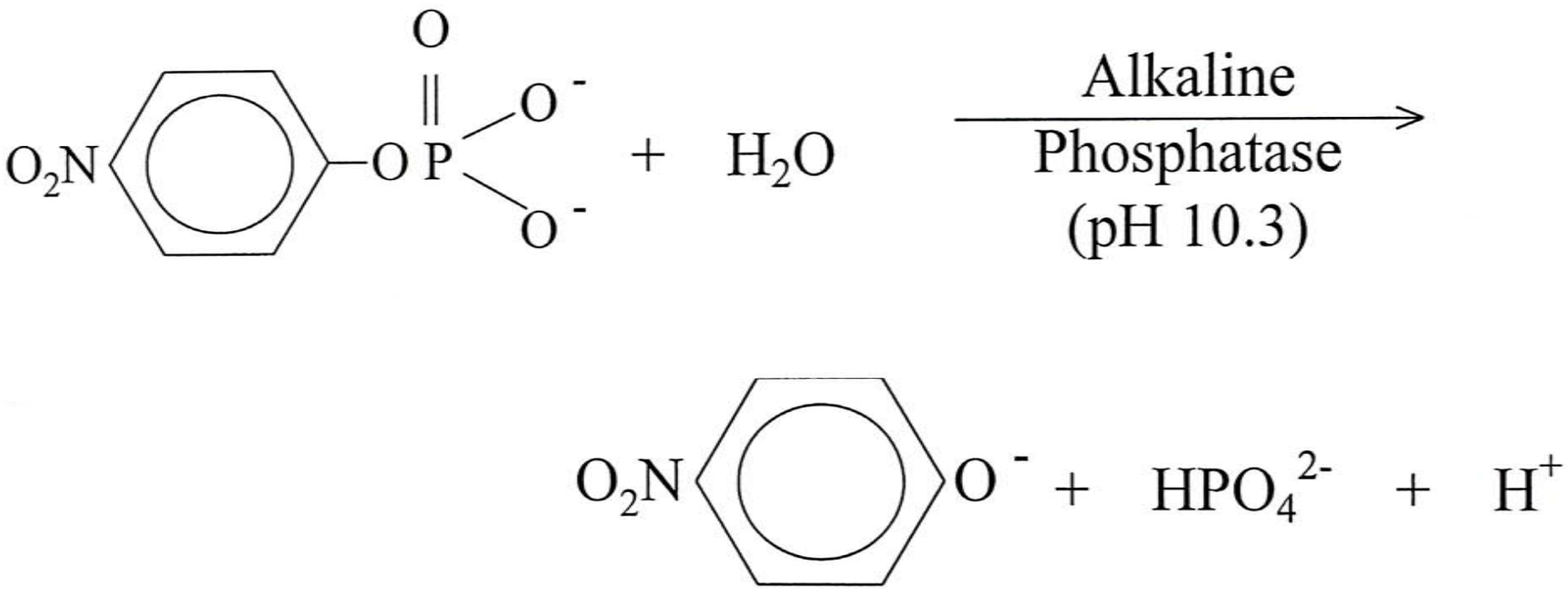


Figure 2.1. Simplified enzymatic equation of p-nitrophenyl phosphate by alkaline phosphatase.

p-Nitrophenyl phosphate (pNPP) Substrate Solution

pNPP substrate was prepared one hour before used. Every tablet of Sigma Diagnostics 104® phosphatase substrate tablet (40 mg of pNPP per tablet) was dissolved in 5 ml of the AMP buffer to give a final concentration of 8 mg/ml. It was allowed to stand for 1 hour and shade vigorously before use.

2.1.3.b Procedure

Plasma samples stored at -70°C were thawed and kept at room temperature. 20µl of each test samples were pipetted into 96 well plates. Reaction was started by adding 250µl of substrate solution and the change of absorbance at 405 nm were continuously monitored at 1 min interval with Dynatech MR5000 Microplate reader. All measurements were preformed in triplicate.

Standard curve of the production of p-nitrophenol (pNp) was constructed using p-nitrophenol standard solution (Sigma Diagnostics 104®, 10 µmol/ml). Serial dilution of 4µmol of pNp standard solution were made by AMP buffer and diluted to 270 µl volume. Absorbance was measured at 405 nm and standard curve was constructed by plotting the amount of p-nitrophenol (µmol) against the absorbance. Amount of pNp formation by the test samples was determined from the standard curve and activity is calculated.

2.1.4 Plasma Bone ALP measurements

Thoughtout the study periods, 3 methods were employed for the plasma bone specific ALP activity measurement. Two methods were based on the Wheat-germ Lectin specific binding of the BALP but using different buffer and autoanalysor system. Methods adopted in Orthopedics and Tramatology Department previous using ABBOTT VP system and A-gent® Alkaline Phosphatase reagents (ABBOTT Method) while samples measured by the Chemical Pathology Department are using MIRA COBAS system with ROCHE ALP IFCC reagent (MIRA COBAS Method).

The wheat germ lectin (WGL) binding assay for BALP measurement was described by Rosalki and Foo (1984). The original method required two-step incubation with a pre-treatment of Triton X-100 provided that serum sample demonstrated the presence of biliary ALP. The pre-incubation was time-consuming and would complicate the calculation of bone-specific ALP activity. The original authors subsequently modified their method so that only one step incubation would be necessary for the precipitation of BALP (Rosalki and Foo, 1986). The modified method was adopted in both departments.

Another newly adopted method in Orthopedic and Tramatology Department is ALKPHASE-B™ (Metra Biosystems) which is an immonassay in microplate strips format utilizing a monoclonal anti-BALP antibody coated on the strip to capture BALP in the plasma sample. The enzyme activity of the captured BALP is detected with a pNPP substrate.

2.1.4.a Wheat Germ Lectin Precipitation of BALP

2.1.4.a.i Reagent

WGL/Triton X-100 Mixture

0.0275 g of WGL from *Triticum vulgaris* (Sigma) and 0.22g of Triton X-100 (Sigma) were dissolved in 5.5 ml ddH_2O to give a final concentration of 5g/L WGL and 40g/L Triton X-100 working solution. The solution was mixed on a roller plate for at least 30 minutes until total dissolution of WGL.

2.1.4.a.ii Procedure

Plasma samples stored at -70°C were thawed at room temperature. Precipitation of BALP was achieved by mixing 50 µl plasma samples with equal volume of WGL/Triton X-100 solution. The mixtures were incubated at 37°C with continuous shaking for 30 minutes. After incubation, the mixtures were centrifuged at 2000 ×g for 10 minutes at 4°C. Supernatant was taken for the estimation of non-bone ALP activity.

2.1.4.b ABBOTT Methods for plasma BALP activity measurement

ALP activity of the untreated plasma and the supernatant were measured by using ABA-100® procedure of the ABBOTT VP system. 50µl of the water blank, the controls and the test sample were pipetted to the sample cup of the ABBOTT VP system. The machine setting was as follow:

Filters	450/415
Syringe Ratio	1:101
Analysis Time	5 min.
Carousel Revolutions	3
Temperature	37°C
End-point or Rate	RATE
Reaction Direction	Down
Calibration Factor	$\frac{10100}{FilterFactor}$
Zero-adjustment	1800

Using the above instrument settings, syringe plate was filled with A-Gent Alkaline Phosphatase reagent. The probe was positioned in the reservoir, Manual Dispense Switch was used to recycle the reagent through the syringe plate, and then the probe was attached to the end of the boom arm. The program was then run, and ALP activity of the samples were automatically calculated by the analyzer.

BALP activity was calculated from the difference between the total ALP in the untreated sample and the supernatant collected after WGL precipitation as shown below:

Bone-specific ALP (U/L) = Total ALP (U/L) - 2 × Non-Bone ALP (U/L)

2.1.4.c COBAS MIRA Methods for BALP measurement

50µl of water blank, controls and each measured sample were pipetted into the sample vial of the COBAS MIRA system. All vials placed into the sample rack, curette ring was installed into the machine and reagent chamber

was filled with ROCHE ALP IFCC reagent. The machine was then run as the following parameter:

GENERAL		
Measurement Mode		ABSORB
Reaction Mode		R-S
Calibration Mode		FACTOR
Reagent Blank		REAG/DIL
Cleaner		NO
Wavelength		405 nm
Unit		U/L
ANALYSIS		
Post Dil. Factor		NO
Post Conc. Factor		NO
Sample Volume		6.0 µl
Diluent	Name	H2O
	Volume	50.0µl
Reagent Volume		250 µl
Reac. Direction		Increase
Low Test Range		NO
High Test Range		1300U/L
Factor		4607

BALP activity of the samples was calculated as in ABBOTT Methods. Control sera from human (TALP activity: High control 319-387U/L; Low control 75-91) were included in each run.

2.1.4.d ALKPHASE-B Method of BALP measurement

Desired number of Anti-BALP Coated Strips were placed in the Stripwell frame and 125µl Assay Buffer was added to each well. Then 20µl of Standard, Control or sample were pipetted to each well within 30 minutes.

The strips were swirled gently to ensure mixing of sample and buffer. The plate was then incubated at room temperature for 3 hours.

The wash buffer concentrate was diluted 10 times with deionized distilled water. Working substrate solution was prepared within one hour of use by adding each tablet (20 mg of pNPP) into 10 ml of substrate buffer. Mixture was allowed to stand for 30 - 60 minutes and then shaken vigorously before use.

After the incubation, the strips was emptied and each well was washed with at least 250μl working wash buffer for a total of 4 times. The strips were vigorously blotted dry after the last wash. Then 150μl Working substrate solution was added into each well and incubated for 30 min. at room temperature. The reaction was then stop by adding 100μl of 1M NaOH, the absorbance of the plate was read at 405 nm by Dynatech MR5000 Microplate reader. All readings were made within 15 minutes after stopping the reaction.

Standard curve was constructed by the BALP standards (0, 2, 20, 50, 80, 140 U/L) and the BALP activity of the samples was calculated from the standard curve. Controls were included in each run (BALP activity: High Control with 55.4-84.0 U/L; Low Control with 11.1 -16.9 U/L).

2.1.5 Inter-conversion of plasma BALP activity measurement in different methods.

In order to compare the results obtained by different methods, correlation between different methods of BALP activity measurement was constructed. Conversion factor between the ALKPHASE-B method and the COBAS MIRA method was calculated by measuring the BALP activity in 58 plasma samples obtained from the Chemical Pathology Department with both methods. Conversion factor between ALKPHASE-B method and the ABBOTT methods was calculated by measuring 67 plasma samples of our patients with both methods. All BALP activity was expressed in unit compatible to the ALKPHASE-B method.

2.1.6 Statistical Analysis

One Way ANOVA, Student's T-test of independent samples were employed for comparison between groups with 2-tailed significance of 0.05. Data were presented using Boxplot. Correlations between groups are investigated by bivariate correlation model with Pearson correlation coefficients and with 2 tailed significance of 0.05.

ANOVA is an statistical analysis used to determine whether means from two or more samples are drawn from populations with the same mean. For the One-way ANOVA, the groups are defined on only one independent variable. In our analysis, One-way ANOVA is used when the means of more than 2 groups are compared.

The normal reference value of the plasma BALP in local Chinese population is determined according to the recommandation of IFCC and International Committee for standardization in Haematology (ICSH). (Solgery, 1987). For age group N1 and N2, normal references was determined by the range of BALP measurement in these subjects. For group N3, non-parametric method was used to determine the lower (0.025 fractile) and upper range (0.975 fractile) of the reference, which contains the central 0.95 fraction of the reference distribution

All analyses were made by SPSS for Windows (Version 6.0) in PC.

2.2 Alkaline Phosphatase Isozymes Expression in Human Osteosarcoma

2.2.1 *In Vitro* Cultures of Human SaOS-2 and U-2 OS Osteosarcoma cell line

Human SaOS-2 and U-2 OS Osteosarcoma cell lines were purchased from the American Type Culture Collection (ATCC).

2.2.1.a Reagent

Phosphate Buffer Saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g KCl, 1.15 g of Na_2HPO_4 and 0.2 g of KH_2PO_4 in 1L double distilled water. The solution was sterilized by autoclaving at 121°C, 15 psi for 30 min.

Dulbecco's Modified Eagle's Medium (DMEM)

Dulbecco's Modified Eagle's Medium powder was purchased from Sigma. The powder was dissolved in 1 liter of double-distilled water, and the solution was supplemented with 3.7 g of $NaHCO_3$ and adjusted to pH 7.2-7.4. Sterilization was done by filtering through 0.22 μm Millipore HV filter, with the addition of 1 % (v/v) penicillin-streptomycin. (Gibco)

Fetal Calf Serum (FCS)

FCS was purchased from Gibco and was stored at -20°C in 10 ml aliquot until use.

Trypsin/EDTA Solution

Trypsin/EDTA solution was purchased from Gibco. The solution contains 0.25% trypsin and 0.53 mM EDTA (ethylenediamine tetraacetic acid, tetrasodium salt) in Hank's Balance Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}.

Glutamine Stock Solution

200 nM Glutamine stock was prepared by dissolving 0.2922g of L-glutamine into 10 ml of DMEM. The solution was then sterilized by filtering through a 0.22 μm membrane.

Standard Medium

Standard medium referred to the medium supplemented with fetal calf serum, antibiotics and L-glutamine. Ten ml of FCS and 1 ml of L-glutamine stock were added into 89 ml DMEM containing 1% PSN.

Trypan Blue Exclusion test

Trypan blue (Sigma) was dissolved in PBS at a final concentration of 0.1% (w/v) and filtered. An aliquot of cell suspension (10μl) was mixed with an equal volume of trypan blue solution. After mixing, the unstained viable cells were counted by a haemocytometer.

2.2.1.b Procedure

2.2.1.b.i Storage of U-2 OS and SaOS-2

Upon receiving from the suppliers, both cell lines, the U-2 OS (ATCC HTB-96) and SaOS-2 (ATCC HTB-85) were thawed at 37°C within 1 minute. The cells were aspeptically transferred to 10 ml of Standard medium and centrifuged immediately at 1300 × g for 5 minutes. The cell pellet was washed with PBS three times. Finally, the pellet was resuspended in standard medium, seeded inside a 75 cm^2 culture flask (Corning) and incubated at 37°C (5% CO_2 ; 95% Humidity) for overnight. The adherent cells were subcultured when confluent. Part of the cells was taken for cell line maintenance. Cell line was kept at the concentration of 1×10^7 cells/ml in 10% DMSO (Dimethylsulfoxide, Sigma) in standard medium. The cell suspension was frozen stepwisely to -70°C and then stored in liquid nitrogen.

2.2.1.b.ii Subculture of Confluent Monolayer

Confluent monolayer of cells was rinsed twice with PBS. Trypsin/ETDA solution (1 ml for 75 cm^2 flask, 0.5 ml for 25 cm^2 flask) was added to the monolayer and the culture flask was incubated at 37°C for 3 minutes. To stop the trypsinization, 5 ml of standard medium was added. The cells were dispersed by repetitive pipette against the monolayer. The released cells were aspirated out. The flask was rinsed with the standard medium and pooled to the released cells. The cells were pelleted at 1500 ×g for 10 minutes and the pellet was washed by plain medium. Finally cells were resuspended in standard medium and viable cell number was counted under hemocytometer

by trypan blue exclusion test. SaOS-2 cell line was maintained by seeding 0.5 $\times 10^6$ cells and U-2 OS cell line by seeding 0.3 $\times 10^6$ cells to 25 cm^2 flask.

2.2.2 Protein Assay

Protein concentration was measured by the Bio-Rad Protein Assay based on the method of Bradford. Microtiter plate protocols of both the standard assay and microassay were adopted.

2.2.2.a Standard Assay

2.2.2.a.i Reagent

Protein standard for standard assay

Protein standards were prepared with bovine serum albumin (BSA) (Sigma) dissolving in double distilled water to obtain protein concentration range from 0.05 mg/ml to 0.5 mg/ml.

Diluted dye reagent for standard assay

Protein Assay Dye Reagent Concentrate (Bio-Rad) was diluted 4 fold with double distilled water and filtered through Whatman # 1 filter to remove particulate.

2.2.2.a.ii Procedure

Ten µl of each standard and sample solution was pipetted into separate microtiter plate wells and 200µl diluted dye reagent was added to each well. The samples and reagent were mixed thoroughly and incubated at room temperature for at least 5 minutes before reading absorbance at 595 nm.

2.2.2.b Microassay

2.2.2.b.i Reagent

2.2.2.b.ii Protein standard for Microassay

Protein standards were prepared with BSA by dissolving in double distilled water to obtain protein concentration range from 8 to 80µg/ml.

2.2.2.b.iii Procedure

160µl of each standard and samples solution were pipetted into separate wells and 40µl of dye reagent concentrate was added into the wells. The samples and the dye were mixed and incubated at room temperature for 5 minutes. Aborbance was measured at 595 nm.

2.2.3 Extraction of ALP from the cultured osteosarcoma cells

The method of extraction of the membrane bound ALP from the cultured cell were modified from the original methods described by Nakamura *et al.*(1988) and Belland *et. al.* (1993).

2.2.3.a Reagents

Cell Washing Buffer (pH 7.6)

Cell washing buffer contains 10 mM of Tris-HCl, 1 mM of $MgCl_2 \cdot 6H_2O$, 0.1 mM of $ZnCl_2$ and 150 mM of NaCl. Solution was prepared in double distilled water and titrated with 1M NaOH to pH 7.6 at room temperature.

ALP Extraction Buffer (pH 7.6)

The ALP extraction buffer contains 100 mM Tris-HCl, 0.1mM of $MgCl_2 \cdot 6H_2O$, 0.02 mM of $ZnCl_2$, and 1% Triton X-100 (w/v). Buffer was prepared with double distilled water and pH adjusted by 1M NaOH to pH 7.6 at room temperature.

2.2.3.b Procedure

Solubilization of ALP

Confluent SaOS-2 cells and U-2 OS cell adherent to the walls of the flask were removed with the help of a rubber policeman. The cells were washed by 2 ml washing buffer and centrifuged at 1500 ×g for 5 mins. The supernatant was discarded and the pellet washed twice with the buffer. The pelleted cells were then resuspended in 1 ml of the extraction buffer. The

resuspension was then undergo a freeze and thaw cycle and then sonicated for 1 minute at a constant low setting using a Beckman Sonic 2000 sonicator on ice. The mixture was rocked gently at 4°C for 90 minutes and the protease inhibitors: leupeptin (5μmol/L), pepstatin A (25μmol/L) and PMSF (0.01%, w/v) were added.

Phosphatidylinositol phospholipase C (PI-PLC) release of ALP

1 Unit of PI-PLC enzyme (Boehringer) with specific activity of 20U/332μl was added to every ml of the cell lysate. The mixture was incubated at 37°C with continuous shaking for 20 hours, followed by centrifuged at 14,000 ×g using Beckman Optima TLX Ultracentrifuge for 10 min. at 4°C. The supernatant was collected and the ALP activity of the released enzyme preparation was measured.

2.2.4 ALP extraction from Human Liver, Placenta and Osteosarcoma Tissue

Membrane preparations from the human tissues were prepared by a modification of the methods of Doellgast *et al.* (1977) and Raymond *et al.* (1991).

2.2.4.a Reagent

Saline

0.9 g of NaCl was dissolved in 100 ml of double distilled water.

Homogenate Buffer

Homogenate buffer contains 250mM sucrose, 5mM $MgCl_2$, 24mM KCl and 50mM Tris-HCl. Buffer was prepared using double distilled water and the pH was adjusted by using 1 M NaOH to pH 7.5. Buffer was kept at 4°C until use.

2.2.4.b Procedure

Tissue Preparation

Placenta: Full-term human placenta was obtained from the Department of Obstetrics and Gynaecology. Weight of the tissue was recorded and the tissue was cut into several pieces with mass approximately 10g per piece. The tissue was washed with saline to remove blood. It was deep frozen in liquid nitrogen and then kept at -70°C until use.

Liver: Human liver was obtained from the post-mortom room within 48 hours of death. After washing with saline, the tissue was cut into small pieces with weight approximately 30 g and was immediately frozen in liquid nitrogen and then kept at -70°C until use.

Osteosarcoma tissue: Human osteosarcoma tissue was obtained during the biopsy of the patients. The tissue was weighted and recorded. It was then frozen in liquid nitrogen and kept at -70°C until use.

Membrane Preparation

Human tissue of various sources were thawed at 4°C overnight, and then chopped into small pieces. The tissue was suspended in equal volume (1 ml / g of tissue) of homogenate buffer. For human osteosarcoma biopsy tissue, since the amount of tissue was small, the tissue was resuspended in 5 ml of buffer. The tissue was homogenized with Polytron (Kinematica®) at full speed on ice for 1 minute. The homogenate was centrifuged at 6000 ×g for 10 minute. The supernatant was collected and filtered through two layers of Miracloth. The filtrate was re-centrifuged at 150,000 ×g for 40 minutes at 4°C. Supernatant was discarded and the pellet was washed with the homogenate buffer (0.3 ml / g of tissue in placenta and liver tissue, 5 ml for osteosarcoma biopsy tissue) and re-centrifuged at 150,000 ×g for another 40 min. at 4°C. The pellet was resuspended with the ALP extraction buffer without Triton X-100. Protein inhibitor leupeptin (5μmol/L), pepstatin A (25μmol/L) and PMSF (0.01%, w/v) were added. Both the ALP activity and

the protein concentration were measured. The crude particulate was stored at -20°C until use.

PI-PLC release of ALP from the crude particulate

Except for the human osteosarcoma biopsy tissue, the crude particulate of human liver and placenta were diluted with the ALP extraction buffer to give a final concentration of 2 μg of protein/ml. Triton X-100 was added to give a final concentration of 1% (w/v). ALP was then released from the membrane preparation as the method described at section 2.2.3.2.

2.2.5 Isoelectric Focusing of ALP

IEF is an electrophoretic technique in which amphoteric compounds are fractionated according to their isoelectric points (pIs) along a continuous pH gradient. The pH gradient is created and maintained by passing an electric current through a solution of amphoteric compound (carrier ampholyte) which have closely spaced pIs, encompassing a given pH range. Agarose IEF is especially suitable for the separation of ALP isozymes since ALP of different origins, although have the same or similar protein sequence, are differ from their carbohydrate moieties (Miura *et al.*, 1994). The difference in their sugar or the nucleic acid composition in the moieties will have influence on the net charge of the protein, providing an addition force for the separation compared to the ordinary zonal electrophoresis.

2.2.5.a Preparation of the agarose IEF gel

Agarose gels were casted on GelBond® film for agarose gel (Pharmacia Biotech). The GelBond was cut into a 12 × 12 cm square. It was then placed on a leveling table with a film of 70% ethanol with the hydrophilic surface up onto the level table. The leveling table was kept horizontal by using a spirit level and the GelBond film was pre-warmed using a hair-dryer.

The 1% agarose gel solution was prepared by dissolving 0.15 g agarose IEF (Pharmacia Biotech), 1.8 g sorbitol (Sigma, Electrophorsis Grade), 1.725

ml glycerol (Pharmacia plusone chemicals, 87% w/v) in 10 ml deionized distilled water in 100-ml conical flask. The solution was warm by boiling water bath until the agarose was dissolved completely. Meanwhile, 0.15 g of Triton X-100 (Pharmacia plusone Chemical) was added in 2 ml of deionized distilled water and warmed in 37°C water bath. The Triton X-100 solution was added into the melted agarose gel solution and mixed by gentle swirling. The temperature of the gel solution was kept at 65°C and 0.715 ml of Pharmalyte® pH 2.5 - 5 and 0.235 ml of Ampholine® pH 3.5 - 9.5 were added into the gel solution and mixed by gentle swirling and avoiding bubble formation. This gel solution was poured and distributed evenly onto the center of the pre-warmed gel support. A tip of the pipette was used to remove any air bubble and to spread the molten gel to the corners of the GelBond. The gel was solidified at room temperature for 15 minutes. Complete solidification was achieved by placing the gel at 4°C in humidity chamber.

2.2.5.b Samples Preparation

Heat inactivation of ALP was employed for further differentiation of various kinds of ALP isozymes after separation on IEF. Heating of the sample at 56°C for exactly 6 min. will preferable destroy the most heat sensitive BALP while heating at 65°C for 10 minutes will essentially destroy all form of ALP except the placental and placental-like ALP isozyme (Moss, 1982).

Before running, 100µl of each sample was incubated at 56°C and 65°C water bath for 6 minutes and 10 minutes respectively. After the incubation, all samples were centrifuged at 1000 ×g for 2 minutes to remove any precipitation. The supernatant and the untreated sample were subjected to separation by IEF.

Each samples was diluted with 1% Triton X-100 solution to obtain a final ALP activity of 100 U/L before the separation.

2.2.5.c Isoelectric Focusing

IEF was performed by using Multiphor II Electrophoresis System (Pharmacia) with Pharmacia LKB MultiDrive XL as power supplier and Pharmacia MultiTemp III as cooling system.

Thermal circulator was turned on and set to 10°C at least 30 mins before the experiment and the electrophorsis plate was adjusted to horizontal position using a spirit level. Meanwhile, IEF gel was brought back to room temperature. Lightly and thoroughly, the gel was blotted with filter paper (Whatman) to remove the water film on the surface of the gel. Sample application template (a plastic film with 15 separate 4 × 8 mm square holes aligned) was placed on 1.5 cm away from the anode side of the gel. Each samples (10μl) was pipetted into the sample wells and allowed to absorb into the gel. Moreover, protein markers IEF-MIX 3.6-9.3 (Sigma) (Detail listed in Appendix) were added into the first, the middle and the last sample well to guide the pH gradient of the IEF gel. Complete absorption of the samples was achieved in around 10 minutes.

Two electrofocusing electrode strips (Pharmacia) was cut into 11 cm long strips. The cathode strips was saturated with 1M NaOH while the anode strip soaked with 1M H_3PO_4. Both strips were blotted thoroughly with paper towel and were placed onto the gel surface. The setting of the gel is shown in Figure 2.2

The gel was placed on the electrophorsis plate, with a thin film of 70% ethanol between the gel and the plate to ensure efficient cooling. The gel was then run in condition with following parameters:

Prefocusing condition:	Voltage	400V
	Current	Maximum
	Power	4W
	Time	15 minutes

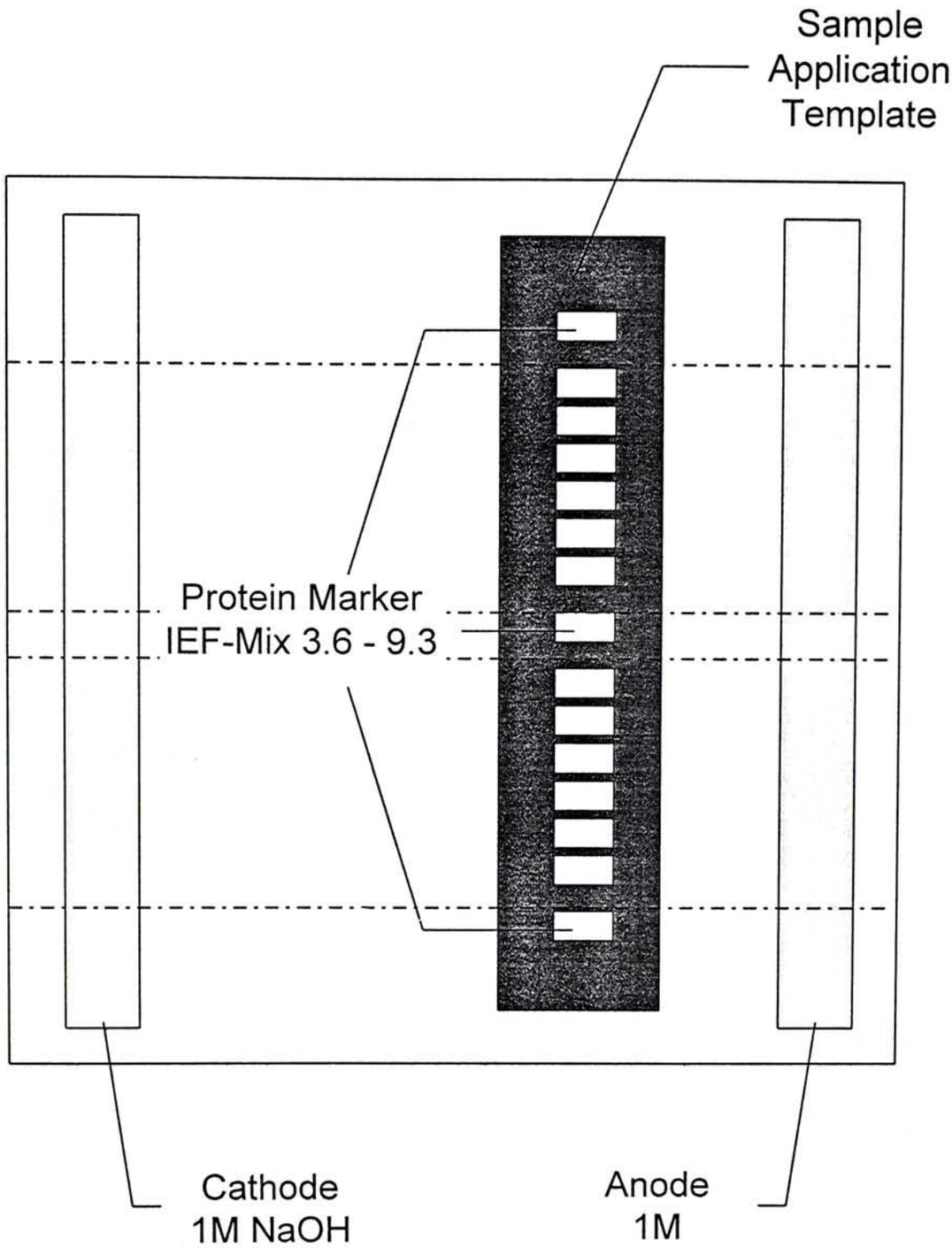


Figure 2.2. Alignment of the Agarose IEF gel

Focusing condition:	Voltage	800V
	Current	Maximum
	Power	5W
	Time	1800 Vhr
Refocusing Condition	Voltage	1500V
	Current	Maximum
	Power	Maximum
	Time	5 minutes

In the prefocusing condition, maximum power of 4W and voltage of 400V with current of around 20 mA were reached initially with gradual decrease of the current during the run. In the focusing condition, voltage gradually increased with the corresponding decrease in the power and current. In general, in the end of the run, the voltage maintained at 800V with Current and Power of around 0.8 mA and 1W respectively. During the run, it was necessary to interrupt the separation and blot the electrode strips with filter paper regularly.

2.2.5.d Protein Detection

Immediately after the focusing, the electrode strips are removed and the gel was cut along the dotted line as shown in figure 2.2 so that the protein marker can be stained by Coomassie Blue solution.

2.2.5.d.i Reagent

Fixing Solution

Fixing solution was prepared by disolving 100g of trichloroacetic acid (Sigma) and 10 g sulphosalicylic acid (Merck) in distilled water and was made up to 1 liter.

Destaining Solution

350 ml of 95% ethanol and 100 ml acetic acid were mixed and made up to 1 liter with distilled water.

Coomassie Blue Staining Solution

1.5 g of Coomassie Blue R 250 (Merck) was dissolved in 500 ml of destaining solution by stirring. The solution was then filtered through filter paper before used.

2.2.5.d.ii Procedure

The gel subjects to the coomassie blue staining was fixed by soaking with the fixation solution for 10 minutes. The gel was then washed with 95% ethanol and dried to a thin film with hair-dryer. The dried gel was stained in the Coomassie Blue staining solution for 5 minutes and then destained in several changes of destaining solution until clear background was obtained. The destained gel was completely dried in 50°C oven overnight. Proteins were stained in dark blue colour.

2.2.5.e Visualization of ALP Isozyme

The ALP separated in the IEF gel was directly visualized by its own enzyme activity. 2.44 mg of α-naphthyl phosphate (Sigma) and 3.76 mg of 4-aminodipenylamine diazonium sulfate (Variamine Blue-RT salt, Sigma) were dissolved in every ml of AMP buffer to give a final concentration of 4.55 mM and 6 mM. This enzyme visualizer was applied onto the gel surface at 37°C in dark for 2 - 24 hours to allow colour development depending on the initial enzyme activity. ALP catalyzes the hydrolysis of α-naphthyl phosphate and forms α-naphthol which in turn couple with the diazonium salt to form a reddish-brown insoluble product. After the colour development, the gel was destained using destaining solution and dried in 50°C oven overnight.

For levamisole inhibition staining, AMP buffer containing 0.06 mM levamisole was used for preparation of the enzyme visualizer. The gel was usually kept overnight at 37°C in dark.

2.2.6 Biochemical Differentiation of ALP expressed in Human Osteosarcoma

Themodenaturation and specific inhibition by amino acid are the classic separation of the ALP isozyme, especially for the confirmation of the presence of placental ALP, which is resistant to heat treatment (Whitby and Moss, 1975)

2.2.6.a Thermodenaturation of ALP

ALP samples from the placenta and the two human osteosarcoma cells line U-2 OS and SaOS-2 were heated at 56°C and 65°C at 0, 0.5, 1, 2, 5, 10 and 15 minutes in heat block. After heating, the samples were immediately chilled on ice and TALP activity of the samples was measured. The degree of inactivation is expressed in percentage of activity remained after heat treatment compared with the unheated sample.

$$\text{Percentage of remained activity} = \frac{\text{ALP activity after heat treatment}}{\text{ALP activity in the untreated sample}}$$

2.2.6.b Amino acid inhibition of ALP

Levamisole and L-phenylalaine were used as inhibitors of the ALP isozyme. Levamisole is an specific inhibition of the tissue non-specific ALP while L-phenylalaine at a concentration of 5 -10 mM, inhibited 80% of the placental and intestinal ALP activity with little effects on the bone and liver ALP activity. (Harris, 1989; Moss, 1986)

AMP buffer containing different concentration of inhibitors was used to prepare the pNPP substrate solution for ALP activity measurement. For levamisole, substrate solution containing final concentrations of 0, 0.5, 1, 2.5 and 5 mM of Levamisole were used for the measurement.

For L-phenylalaine, substrate solution containing final concentration of 0, 1, 5, 10 and 20 mM of L-phenylalaine were used for the ALP activity

measurement. Again, the degree of inhibition is expressed in percentage of activity remained after addition of inhibitors.

$$\text{Percentage of remained activity} = \frac{\text{ALP activity after inhibition}}{\text{ALP activity in the untreated sample}}$$

2.2.7 Immunohistostaining of placental ALP in human Osteosarcoma

2.2.7.a Reagent

Poly-L-Lysine solution

0.1% (w/v) poly-L-lysine (Sigma) solution was prepared by dissolving 0.1g of poly-L-lysine in dd H_2O. The poly-L-lysine solution was filtered throught a 0.22 μm sterile membrane (Millipore) and stored in 4°C.

Neutral Buffered Formalin Solution

100 ml of Formalin (40% w/v formaldehyde) was mixed up with PBS to a final volume of 1L.

ABC Regent

The ABC reagents were prepared by mixing 2 μl of reagent A (Avidin DH) and 2 μl of reagent B (Biotinylated Horseradish Peroxide H) into 1ml of PBS. The ABC reagent was allowed to stand 30 mins in 4°C before use.

0.1m Tris Buffer (pH 7.2)

6.05g of Tris-Base and 9 g of NaCl was dissolved in 900ml of ddH_2O. pH of the solution was adjusted with 1M HCl and the solution was made up to 1 L.

Diaminobenzidine tetrahydrochloride (DAB) colour dievelopment

DAB concentrate was prepared by dissolving 1mg of DAB into 1 ml ddH_2O. Just before use, 0.02ml of DAB concentrate and 3.4μl of 30% H_2O_2 (Merck) were added into 1 ml of 0.1M Tris Buffer (pH 7.2).

2.2.7.b Preparation of human osteosarcoma cell line

Round glass slide with diameter of 1 cm were clean by soaking in acetone for 15 mins followed by 0.2M HCl for another 15 mins. The glass slid were raised with ddH_2O and baked at 180°C overnight. The hot glass slid were cooled down and than coated with the poly-L-lysine solution for 15 mins. The coated slid were raised with steriled ddH2O for 15 min and then air dried under UV light.

Both U-2 OS and Sa OS-2 cell were cultured according to previous sections. After confluent, the cells were released by trypsinization. The released cells were resuspened in plain medium to a concentration of 1×10^5 cells/ml. 200μl of the cell suspension were seeded onto the glass slid placed in a 25mm culture dish for 30 mins. 2 ml of plain medium were added into the culture dish and placed in the incubator overnight. The medium was then removed and the seed cells were washed with PBS followed by a 15 mins fixation using neutral buffered formalin. The seeded cells were then washed in ddH_2O for 5 mins and follow the immunostain procedures.

2.2.7.c Preparation of human osteosarcoma tissue

Seven human osteosarcoma tissues embedded in paraffin were obtained from the Department of Anatomical and Cellular Pathology, CUHK. 7 μm paraffin sections were obtained by microtome. Routine Hematoxylin and Eosine stain were applied to all sections to ensure the presence of osteosaroma tissue within the sections.

The paraffin sections were de-waxed in 2 wash of xylene, each for 5 mins, and then hydrated with decreasing concentration of ethanol and finally rinised in running water. The sections were then ready for immunostaining.

Cytosection of human placenta tissue and human liver tissue section were used as the positive and negative control for the immunohistostaining.

2.2.7.d Immunohistostaining

All sections were quenched for endogenous peroxidase by incubating in 0.02% H2O2 for 10 mins in room temperature. The section were then block with 1% Bovine Serum Albumin (BSA) in PBS (BSA/PBS) for 20 min. After blocking, the 1% BSA/PBS were wraped dry. Primary antibody 1:20 (Rabbit monoclonal antibody against human placental ALP, Immunodiagnostika & Biotechnologie, produktion & Vertriebs GmbH, Germany) diluted in 0.1% BSA/PBS were applied on the sections incubated at 4°C overnight.

After the incubation, the primary antibody was wrap off and the sections were washed 3 times by soaking in 0.1% BSA/PBS solution. The sections were then incubated with Biotinylated 2nd antibody (against rabbit IgG) provided by Vectastain® ABC kit for 30 mins. After incubation with the 2nd antibody, the sections were washed 3 times in 0.1% BSA/PBS (each 5 mins) and then incubated with the ABC reagent for another 1 hour.

The ABC reagents were removed by washing with 3 fold of 0.1% BSA/PBS and the DAB colour development were applied on the section and incubated for 10 mins in dark. After the development of reddish brown colour, the sections were washed with running water and counterstain with Hemotoxylin. Sections were then dehydrated with increasing concentration of ethanol and finally in xylene and then mounter with a coverslid in histomount.

Chapter Three: Results

3. Plasma BALP measurement in Osteosarcoma patients

3.1 General Information of the Patients

Between 1990 to May 1997, 49 patients who have been diagnosed with primary osteosarcoma were recruited for the study. All the patients were referred to the Department of Orthopaedics & Traumatology, Prince of Wales Hospital for their management.

Diagnosis of osteosarcoma was based on clinical, radiographic findings and biopsy. Biochemical investigations included blood tests: CBP, ESR, LDH, plasma calcium, phosphate and alkaline phosphatase. Radiological investigations included the CXR, X-ray of affected extremity or pelvis, bone scans and MRI. These investigations helped to stage the disease and detect any metastasis. The diagnosis was confirmed by histology of the biopsy tissue.

3.1.1 Age and Sex Distribution

The details of all osteosarcoma patients are listed in Table 3.1. The age distribution of the 49 patients with proven osteosarcoma is shown in Figure 3.1. Patients' age ranged from 9 to 61, with a mean of 20.8. Highest incidence of osteosarcoma occurred at age 18 (12.24%). A peak was observed in adolescence, 65.3% of the patients under 20 years of age. There was a male predominance, with 65.3% male patients and 34.7% female patients.

3.1.2 Sites

In 85.7% of patients, the primary site of osteosarcoma was in the long bones. There were 21 cases in the femur, 17 cases in the tibia and 4 cases in the humerus. In the remaining 7 cases, the affected bone was the pelvis. Metastases at admission were detected in 5 patients with sites of metastasis at lung and ribs. One of the patients with primary lesion in the humerus had multiple metastasis in the skull, cervical spine, ribs, pelvis and the femur.

3.1.3 Treatment and survival rate

All except 2 patients with low grade sarcoma and 1 patient who refused treatment, patients received neoadjuvant chemotherapy according to a modified Rosens T10 protocol developed in 1974 (Rosen *et al.*, 1974). Preoperative regime included the use of cisplatinum, adriamycin and high dose methotrexate as shown in Table 3.2. Most patients received 2 to 4 courses of pre-operative chemotherapy.

Wide excision of the lesion and allograft reconstruction was performed after week 10 of pre-operative chemotherapy. Rotationplasty was performed in one patient. For patients with local recurrence, amputation was performed to ensure the complete removal of the tumor. Post-operative chemotherapy was begun 2 weeks after the surgery with a similar protocol as the pre-operative chemotherapy. Patients with favorable response toward the chemotherapy (Huvo's III and IV based on the histological examination on the excised tumor specimen received) repeated treatment with week 1-4 cycles for three times. Patients with unfavorable response (Huvo's I & II) were switched to another salvage protocol listed in Table 3.3.

Follow-up investigations after the completion of the chemotherapy included: Blood test in every 3 months in the first 4 years and then half-yearly from the 5th year onward; CT Thorax for every 4 to 6 months in 1-4 years and than annually from 5th year; Bone Scan biannually in the first 2 years, annually in the 3rd and 4th year.

The longest follow-up time for our patients is 7 years with mean follow-up time of 2.5 years. The 2 years survival rate is 64.5%. Main cause of death was local recurrence and metastasis. One patient died from adriamycin toxicity during chemotherapy. The average time of recurrence is 10 months and average time for metastasis (exclude metastasis detected at admission) is 28.68 months.

Patient Code	Sex /Age	Site	Date of Diagnosis	Metastasis	Recurrence	Die of disease	Follow-up Period
BWY	F/12	Femur	14/2/96	Admission			15 months
KSW	F/14	Femur	20/3/96	Admission		5 months	
CML	F/18	Pelvis	12/94		28 months		28 month
CCK	F/18	Tibia		14 months		24 months	
TCY	F/18	Tibia	11/9/91	13 months			6 Year
KP	F/22	Tibia		12 months		19 months	
HSF	F/26	Femur	9/8/96	1 month			8 months
KCY	M/10	Tibia	29/8/91	9 months			6 Years
THL	M/10	Tibia	8/96	Admission	5 months	11 months	
SKW	M/11	Femur	13/9/91	30 months		35 months	
WCK	M/12	Tibia	11/3/92	9 months			5 Years
YCY	M/13	Tibia	29/9/96	8 months			10 months
CLY	M/14	Pelvis	20/1/92	10 months			5 Years
CSL	M/15	Humerus	14/7/95	Admission			10 months
PTM	M/16	Femur		14 months	9 months	24 months	
CHW	M/17	Femur	14/795	10 months	18 months		22 months
SHL	M/17	Tibia	24/12/96	Admission			5 months
CHL	M/18	Femur		12 months		16 months	
YKC	M/18	Pelvis		6 months	6 months	11 months	
AKM	M/18	Pelvis	15/7/92	13 months		13 months	
MCM	M/21	Pelvis		6 months	2 months	9 months	
SC	M/24	Tibia		6 months	4 months	9 months	
LMC	M/30	Pelvis	21/9/92	15 months		26 months	
TYC	M/38	Femur	24/4/92	6 months			5 Years
LFH	M/40	Femur	2/11/92	24 months		36 months	
WCW	M/45	Femur	8/96	5 months			9 months
LDK	F/11	Tibia	9/6/96				11 months
YLP	F/12	Humerus					5 Year
LYM	F/12	Tibia	11/7/95				2 Years
MLK	F/12	Femur	14/1/97				4 months
KSP	F/18	Humerus	23/9/91				6 Years
CY	F/30	Tibia	12/89				7 Years
CMS	F/31	Femur	2/4/97				1 month
LW	F/36	Tibia	2/9/91				6 Years
CKL	F/9	Femur	27/12/96				6 month
LSF	F/9	Femur	18/3/97				2 months
YSM	M/11	Femur	1990				6 Years
WTF	M/11	Femur	29/12/94				28 months
TCWJ	M/12	Pelvis	16/12/92			24 month	
HWH	M/12	Femur	29/1/97				4 months
LWS	M/13	Tibia	1993				3 Years
LKH	M/14	Humerus					4 Years
LCK	M/17	Femur	7/5/96				2 Years
TCC	M/20	Tibia	1/10/92				5 Years
AMW	M/21	Femur					4 Years
MSK	M/23	Tibia					5 Years
TCW	M/28	Tibia	10/94				2.5 Years
LCS	M/51	Femur	20/12/96				5 months
HHW	M/58	Femur	11/7/96				9 months

Table 3.1. General information of the osteosarcoma patients.

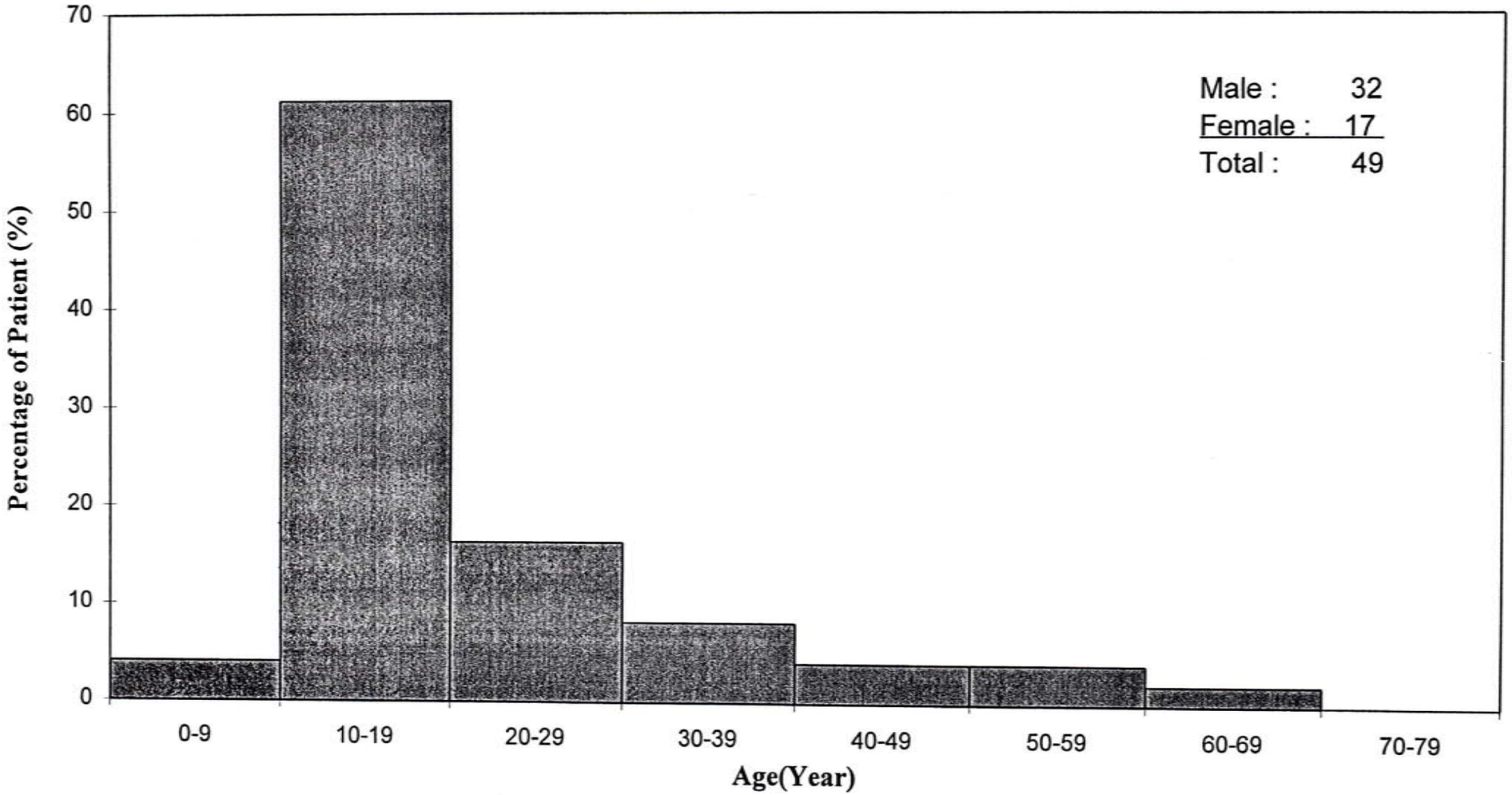


Figure 3.1 Age Distribution of the patients

Week	Treatment
1 Day 1 Day 1 - 2	Cisplatinum 100mg/m^2 Adriamycin 30 mg/m^2
3	Methotrexate 8 g/m^2
4	Methotrexate 12g/m^2
5 - 8	Repeat weeks 1 - 4

Table 3.2. Pre-operative chemotherapy (Rosen T10) protocol used in Prince of Wales Hospital.

Week	Treatment
16 Day 1 - 3	VP16 100mg/m^2 Ifosfamide 2.5 g/m^2
19 Day 1 Day 1 - 2	Cisplatinum 100 mg/m^2 Adriamycin 30mg/m^2
22 -27	Repeat weeks 16 - 19 for 3 cycle
40 Day 1 - 3	VP16 100mg/m^2 Ifosfamide 2.5 g/m^2

Table 3.3. Post-operative chemotherapy protocol for unfavourable response patients.

3.2 Clinical Significance of Plasma Bone Specific Alkaline Phosphatase (BALP) Activity measurement in Osteosarcoma Patients

3.2.1 Plasma BALP Activity measurement

Two different methods of BALP measurement were used in this study. WGL precipitation assay using A-Gent Alkaline Phosphatase Test reagent and ABBOTT VP system (refer to methodology section 2.14a) were previously adopted in the Orthopeadic and Tramatology Department, Prince of Wales Hospital before the development of ALKPHASE-B ELISA Kit for the BALP isozyme measurement.

In addition, part of the BALP measurement was done in the main laboratory in the Chemical Pathology Department, Prince of Wales Hospital. WGL precipitation assay using ROCHE ALP IFCC reagent and COBAS MIRA system (refer to methodology section 2.14c) was adopted in the Chemical Pathology Department.

All the Bone-ALP measurement units were in U/L, which is defined as μmol of p-nitrophenyl phosphate hydrolyzed per minute. However, since the methodology and reagents were different, the correlation between different methods was established to allow comparison of the data. All the Bone-ALP measurements are finally presented using the unit defined in ALKPHASE-B ELISA kit. The correlation of Bone-ALP measurement between ALKPHASE-B and ABBOTT VP system is shown in Figure 3.2. The correlation of Bone-ALP measurement between ALKPHASE-B and COBAS MIRA system is shown in Figure 3.3.

For the BALP level measured by Wheat germ lectin binding assay measured by the ABBOTT method, the values should be converted by the following equation, which is the best-fitted linear equation obtained on Figure 3.2:

$$\text{ALKPHASE-B} = 0.5498 \times \text{ABBOTT} + 0.8774$$

The correlation (R^2) of values measured by these two methods are 0.9687, showing a valid conversion.

For the BALP level measured by the Chemical Pathology Department, the value should converted to compatible unit by the equation, which is the best-fitted linear equation obtained on Figure 3.3:

$$\text{ALKPHASE-B} = 0.772 \times \text{COBAS MIRA} - 0.5216$$

The correlation (R^2) of values measured by these two methods are 0.9075.

3.2.2 Normal Reference of Plasma BALP determination

Normal subjects from the local Chinese population in different age groups were recruited for the determination of normal reference value of plasma Bone-ALP level. Normal subjects had no past history or present sign or symptoms of liver and metabolic bone disease at the time of recruitment. The Department of Pediatrics, Prince of Wales Hospital, kindly provided the plasma samples of subject with age under 12.

Subjects were divided into 3 groups according to their ages: N1 (age below 12); N2 (age between 12 and 16) and N3 (age above 16). According to the International Federation of Clinical Chemistry (IFCC) and the International Committee for Standardization in Hematology (ICSH) recommendation (Solberg, 1987), the normal reference of plasma bone-ALP level is calculated and summarized in Table 3.4.

Due to the limitation of blood sample collection in Group N1 and N2, normal range of plasma BALP level in this two age groups are estimated by the range of the plasma BALP activity. The normal range of plasma BALP level in N1 is 73.6 to 217.7 U/L with mean value of 128.28 U/L. For group N2, the normal range is 21.7 to 136.8 U/L with mean of 75.31 U/L.

For plasma BALP normal reference determination in group N3, non-parametric methods were used to determine the lower (0.025 fractile) and upper range (0.975 fractile) of the reference, which contains the central 0.95 fraction of the reference distribution. The calculated range is 8.9 to 59.5 U/L with mean of 22.08 U/L.

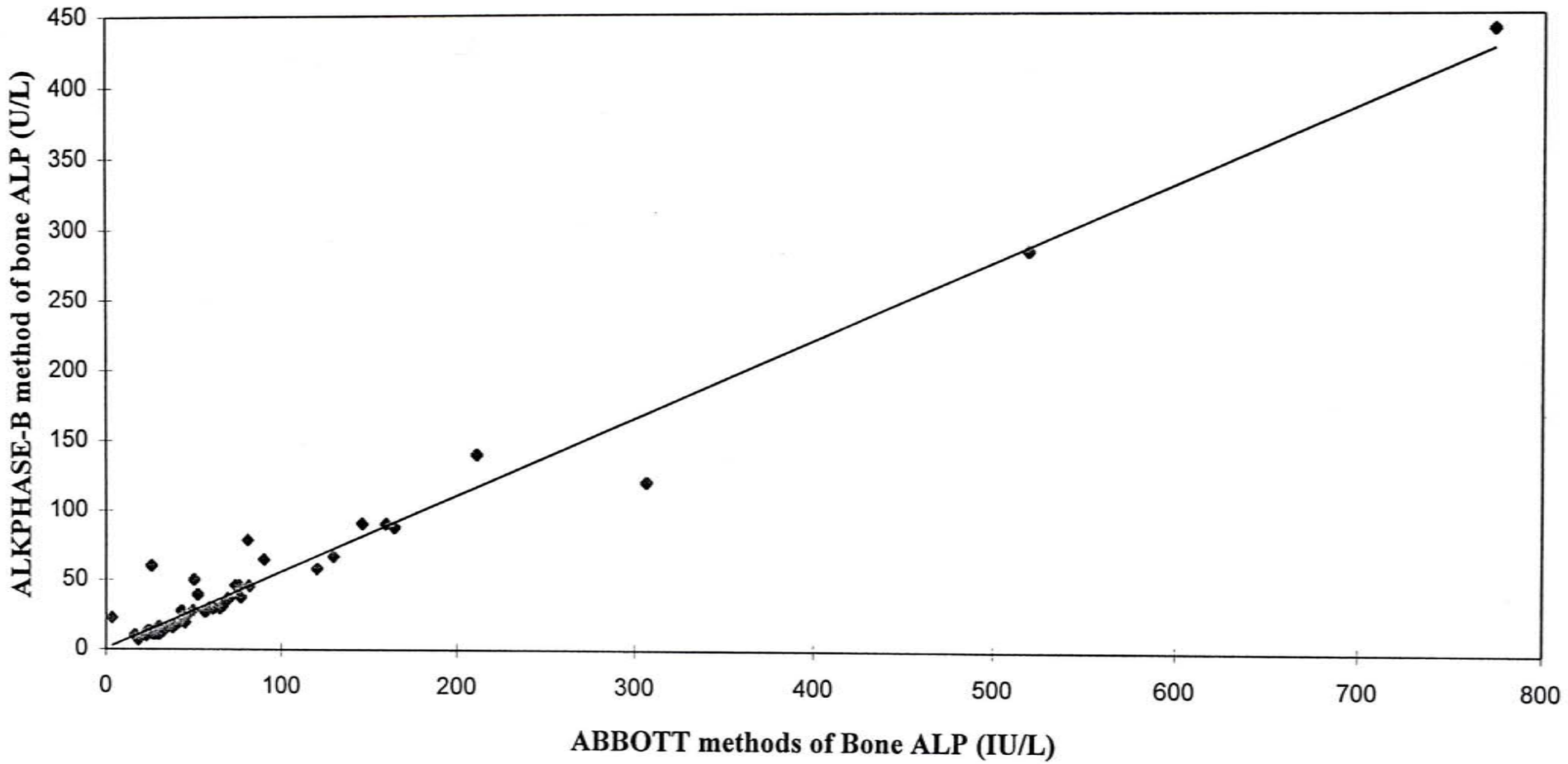


Figure3.2 Correlation between the ALKPHASE-B ELISA methods and the ABBOTT method for Plasma Bone-ALP determination

* 67 human plasma sample were used, $R^2 = 0.9687$

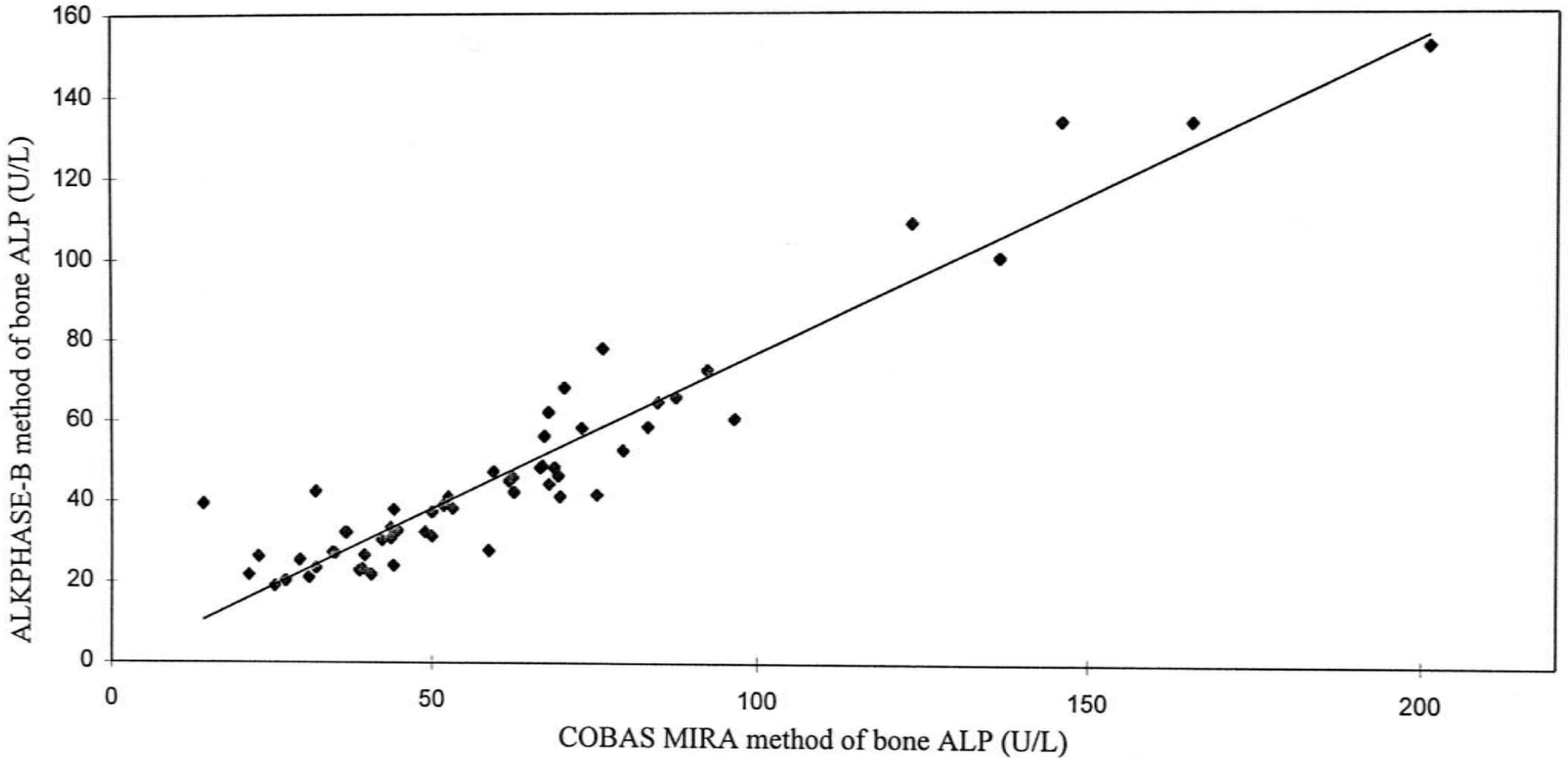


Figure 3.2 Correlation between the ALKPHASE-B ELISA methods and the COBAS MIRA method for BALP determination

*57 human plasma sample were used, R2 = 0.9075

When we compare the plasma BALP level in three groups, using one-way ANOVA Duncan test (Figure 3.4), there is a significant difference between the three groups ($p < 0.05$), with highest plasma BALP level in N1 and N2 and lowest in N3.

3.2.3 Diagnostic value of Plasma BALP measurement in Osteosarcoma

3.2.3.a Plasma BALP level at admission

Blood samples of the osteosarcoma patients were collected during admission, usually the day before the biopsy. Plasma BALP activity was measured by either one method described in the methodology section. Since no treatment was done before the confirmation of diagnosis by biopsy, the plasma BALP level at admission (BALP-Adm) was used to represent the initial plasma BALP level before any treatment. Patients were divided into three groups according to their ages at diagnosis, namely T1 with age below 12; T2 with age between 12 to 16 and T3 with age above 16. The BALP-Adm at these three age groups is shown in Table 3.5-7 respectively.

By using 2-Way ANOVA analysis, BALP-Adm of the patients was compared with the plasma BALP of the normal with respect to both the age group and tumor-bearing factor. Results show that there is significant difference ($p < 0.001$) in the plasma BALP level between tumor bearing and non-tumor bearing, independent of age (Figure 3.5.). However, there is no significant difference ($p = 0.727$) in the plasma BALP level between individual age groups, independent of the tumor-bearing factor.

Moreover, the interaction between age group and tumor bearing factors on the plasma BALP is significant ($p = 0.003$). This implies that between different age groups, the difference in plasma BALP is not the same. As a result, Student's t-test was used to compare the plasma BALP level between the normal subjects and osteosarcoma patients in each individual age group.

Independent-samples Student's t-test was performed between the plasma BALP level of the normal subjects and osteosarcoma patients in

Group	Age	No. of Subject	Male	Female	Plasma Bone ALP (U/L)	
					Lower Range	Upper Range
N 1	Below 12	24	15	9	73.6†	217.7†
N 2	Between 12 - 16	10	4	6	21.7†	136.8†
N 3	Above 16	103	63	40	8.9*	59.5*

Table 3.4 Normal Reference of Plasma Bone-specific Alkaline Phosphatase Level in Local Chinese Population.

* The lower and upper range are determined by the 0.25 and 0.975 fractile

† According to IFCC and ICSH recommandation, since the number of subject is below 40, the range are used as a guide of the reference.

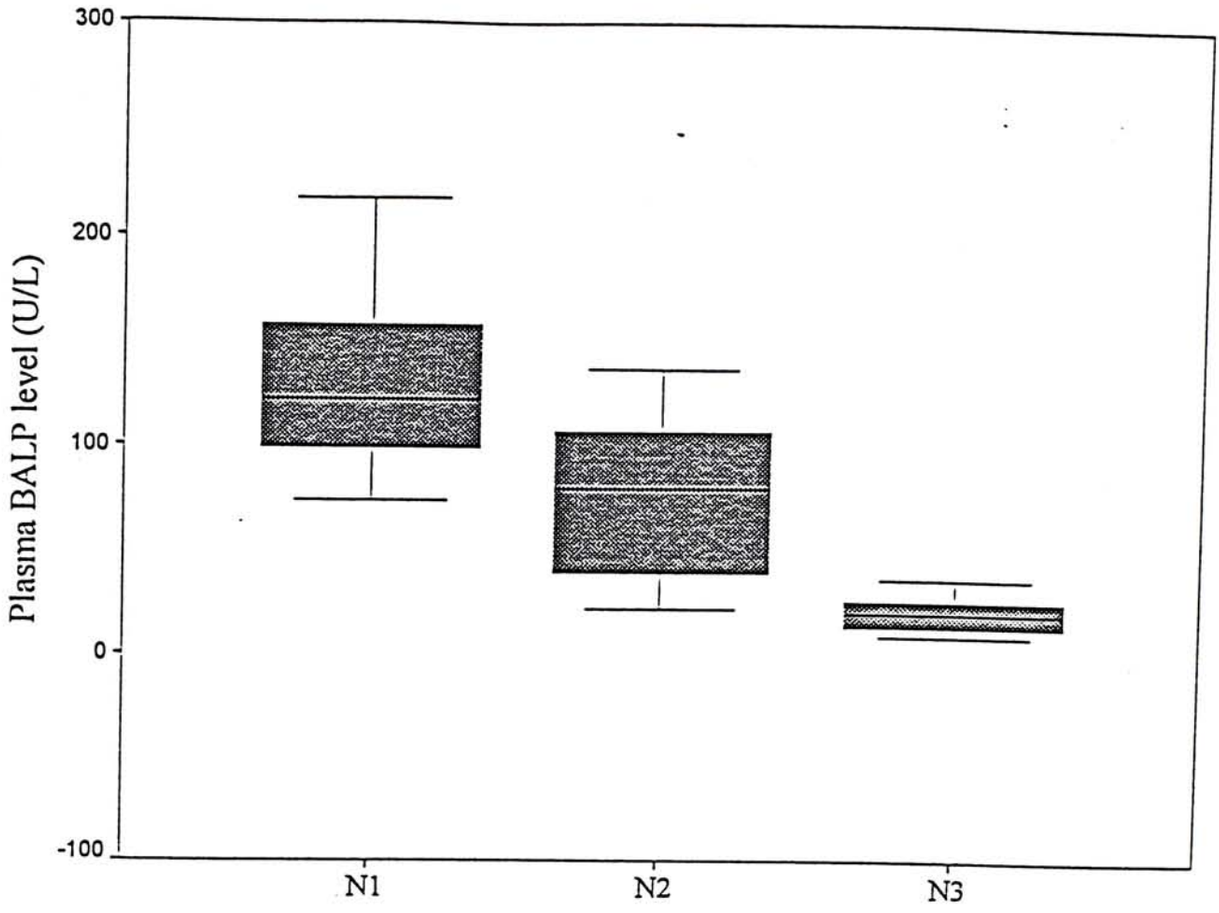


Figure 3.4. Boxplot of plasma BALP level of Normal Subjects in different age group

* N1 = age below 12 (n=23); N2 = age between 12 to 16 (n= 10) and; N3 = age above 16 (n=103).
Analysis done by One-way ANOVA ,with $p < 0.05$

For Boxplot, the upper bar and lower bar represent 95 and 5 percentile of the data respectively; the upper and the lower margin of the box respresent 75 and 25 percentile of the data and the middle line respresents the mean of the data.

different age group. The results are shown in the form of box plot in Figure 3.6-8.

In age group 1 (age < 12), there is no significant difference between the plasma BALP from the normal subjects and the BALP-Adm of the osteosarcoma patients ($p = 0.145$). In group 2 (age 12 -16) and group 3 (age > 16), BALP-Adm is significantly higher than the plasma BALP in normal group, with two tailed significance of $p = 0.006$ and $p < 0.001$ respectively.

3.2.3.b Plasma Total ALP level at admission

Plasma total ALP level at admission (TALP-Adm) was also measured in the admission plasma sample and was recorded in Table 3.8. Student's t-test was used to compare TALP-Adm with total plasma ALP of the normal subjects. We found that the plasma TALP-Adm in osteosarcoma patients is significantly higher ($p < 0.001$) than the plasma ALP of normal subjects. (Figure 3.9.)

Plasma TALP-Adm in different age groups (T1, T2 & T3) were also compared with the age matched normal reference N1, N2 & N3 respectively using Student's t-test. The results were shown in Figure 3.10 - 3.12. In both group T1 and T2, there is no significant difference between the plasma TALP-Adm and the plasma total ALP level, with $p = 0.273$ and $p = 0.096$ respectively. In group T3, plasma TALP-Adm level is significantly higher than the plasma total ALP of the normal group (N3), with $p = 0.002$.

3.2.4 Prognosis Value of Plasma BALP measurement in Osteosarcoma Patients

3.2.4.a Correlation of plasma BALP-Adm with the local relapse of the disease

Patients with the completion of the post-operative chemotherapy and their plasma BALP-Adm were shown in Table 3.9. Seven out of 41 patients experienced local recurrence after the operative removal of the tumor with an average relapse time of 9.7 months. By using Student's t-test, we found that the BALP-Adm of the patients who experienced local recurrence is

Patient Code	Sex/Age	BALP-Adm (U/L)*
CKL	F/9	192.58
LSF	F/9	44.88
KCY	M/10	220.80
TI	M/10	244.84
WL	F/11	99.40
SKW	M/11	271.88
YSM	M/11	566.13

Table 3.5 Plasma BALP level at admission of patients in age group T1 (below 12)

* BALP unit are expressed in ALKPHASE-B unit.

Patient Code	Sex/Age	BALP-Adm (U/L)*
YLP	F/12	521.35
BWY	F/12	102.80
LYM	F/12	107.66
MLK	F/12	97.22
WCK	M/12	290.60
TCWJ	M/12	215.69
HWH	M/12	184.12
LWS	M/13	631.75
YCY	M/13	64.64
KSW	F/14	1057.49
LKH	M/14	326.03
CLY	M/14	178.80
CSL	M/15	203.26

Table 3.6. Plasma Bone ALP level at admission of patients in age group T2 (between 12 to 16)

* Bone ALP unit are expressed in ALKPHASE-B unit.

Patient Code	Sex/Age	BALP-Adm (U/L)*
PTM	M/16	789.23
CHW	M/17	168.72
LCK	M/17	34.54
SHL	M/17	71.68
TCY	F/18	119.78
KSP	F/18	87.99
CML	F/18	702.77
CCK	F/18	691.96
CHL	M/18	319.09
AKM	M/18	494.92
YKC	M/18	837.10
TCC	M/20	81.75
AMW	M/21	289.75
MCW	M/21	790.01
KP	F/22	527.53
MSK	M/23	547.60
SC	M/24	934.37
HSF	F/26	215.38
CY	F/30	624.03
LMC	M/30	115.80
LW	F/36	67.56
TYC	M/38	158.37
LFH	M/40	245.75
WCW	M/45	228.65
LCS	M/51	1745.93
HHW	M/58	28.97
CMS	F/61	444.90

Table 3.7 Plasma Bone ALP level at admission of patients in age group T3 (above 16)

* BALP unit are expressed in ALKPHASE-B unit.

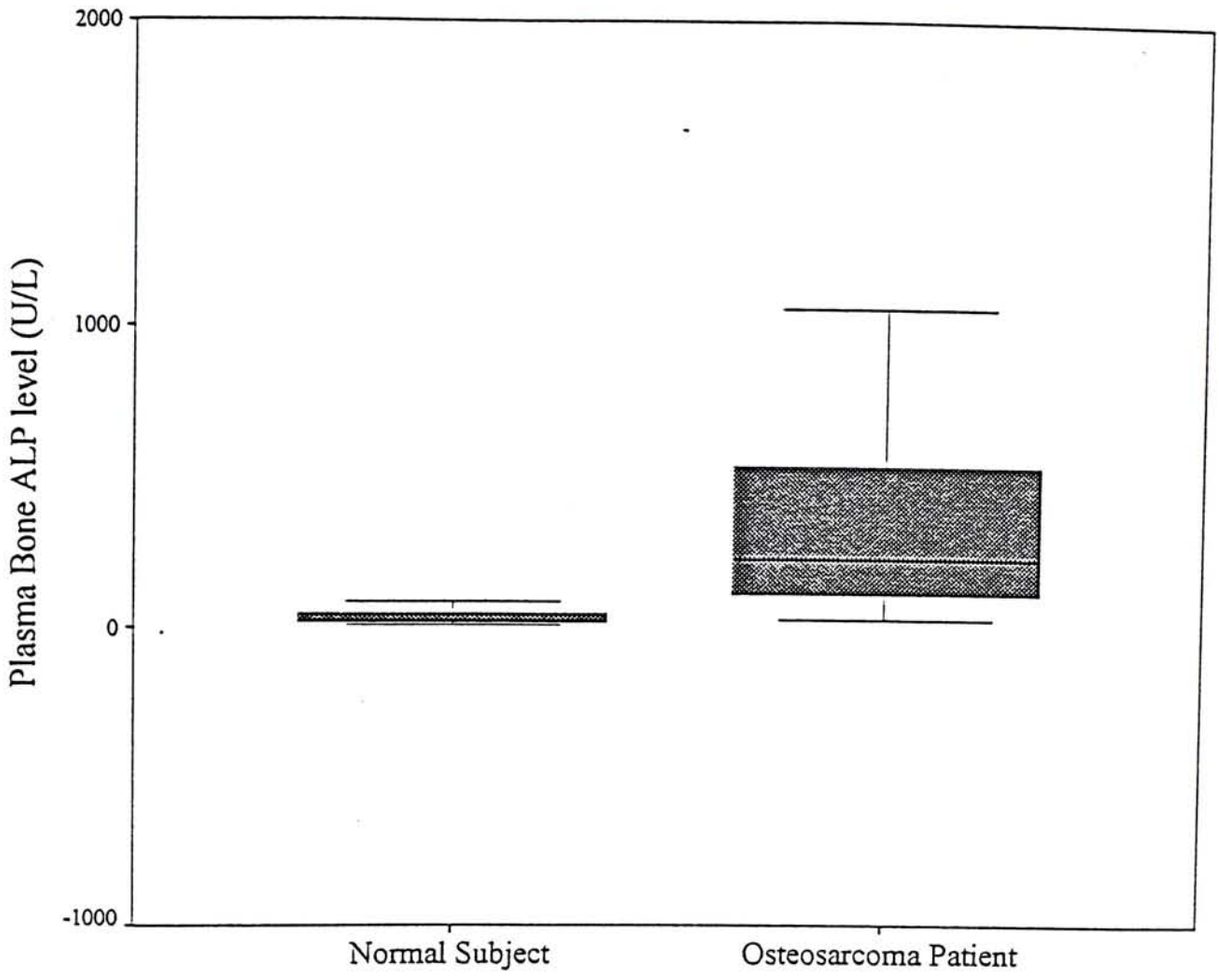


Figure 3.5 Boxplot of plasma BALP level in Normal Subjects and in osteosarcoma patients, independent of age.
Analysis done by 2-Way ANOVA, $p < 0.005$ between the different of the noraml subjects (n =136) and osteosarcoma patients (n = 47).

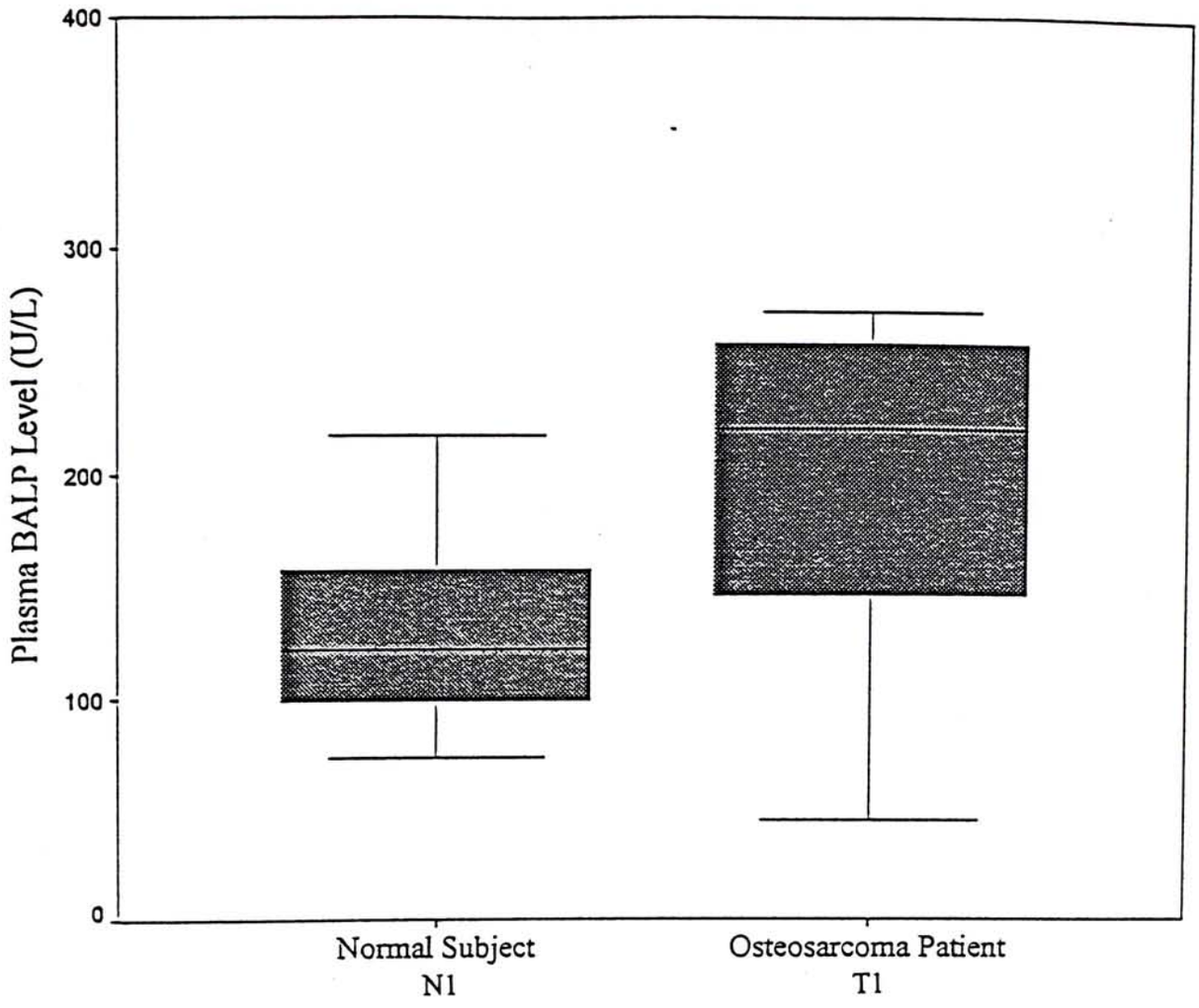


Figure 3.6. Boxplot of Plasma BALP level in Normal Subjects (N1) and Osteosarcoma Patients (T1) with age below 12.
For osteosarcoma patients, the plasma BALP level is the BALP-Adm. Analysis done by Student's t-test, with $p > 0.1$, between the difference of normal subjects N1 ($n = 23$) and the osteosarcoma patients T1 ($n = 7$).

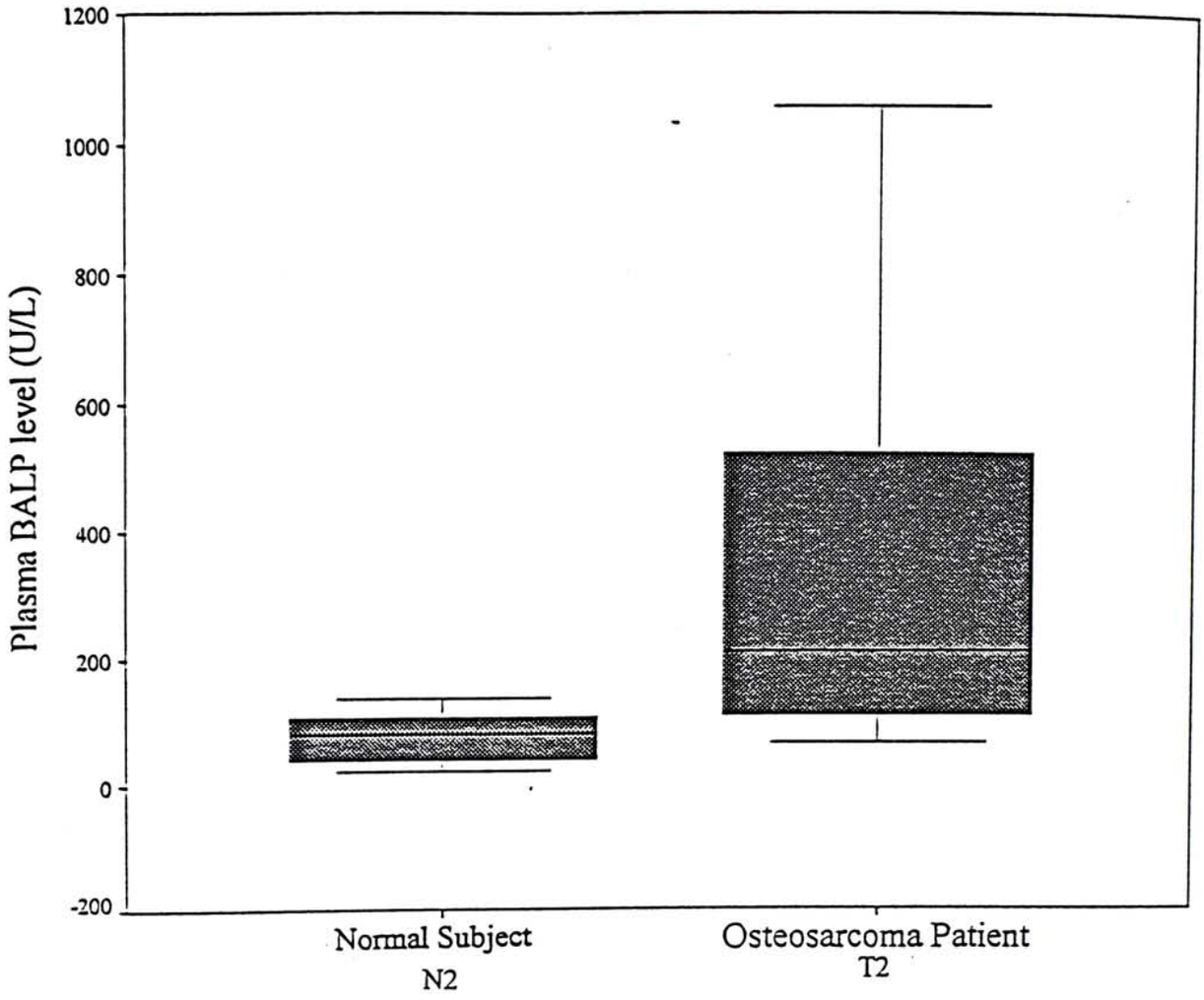


Figure 3.7. Boxplot of Plasma BALP level in Normal Subjects (N2) and Osteosarcoma Patients (T2) with age between 12 and 16.

For osteosarcoma patients, the plasma BALP level is the BALP-Adm Analysis done by Student's t-test, with $p < 0.01$, between the different of normal subjects N2 ($n = 10$) and the osteosarcoma patients T2 ($n = 14$).

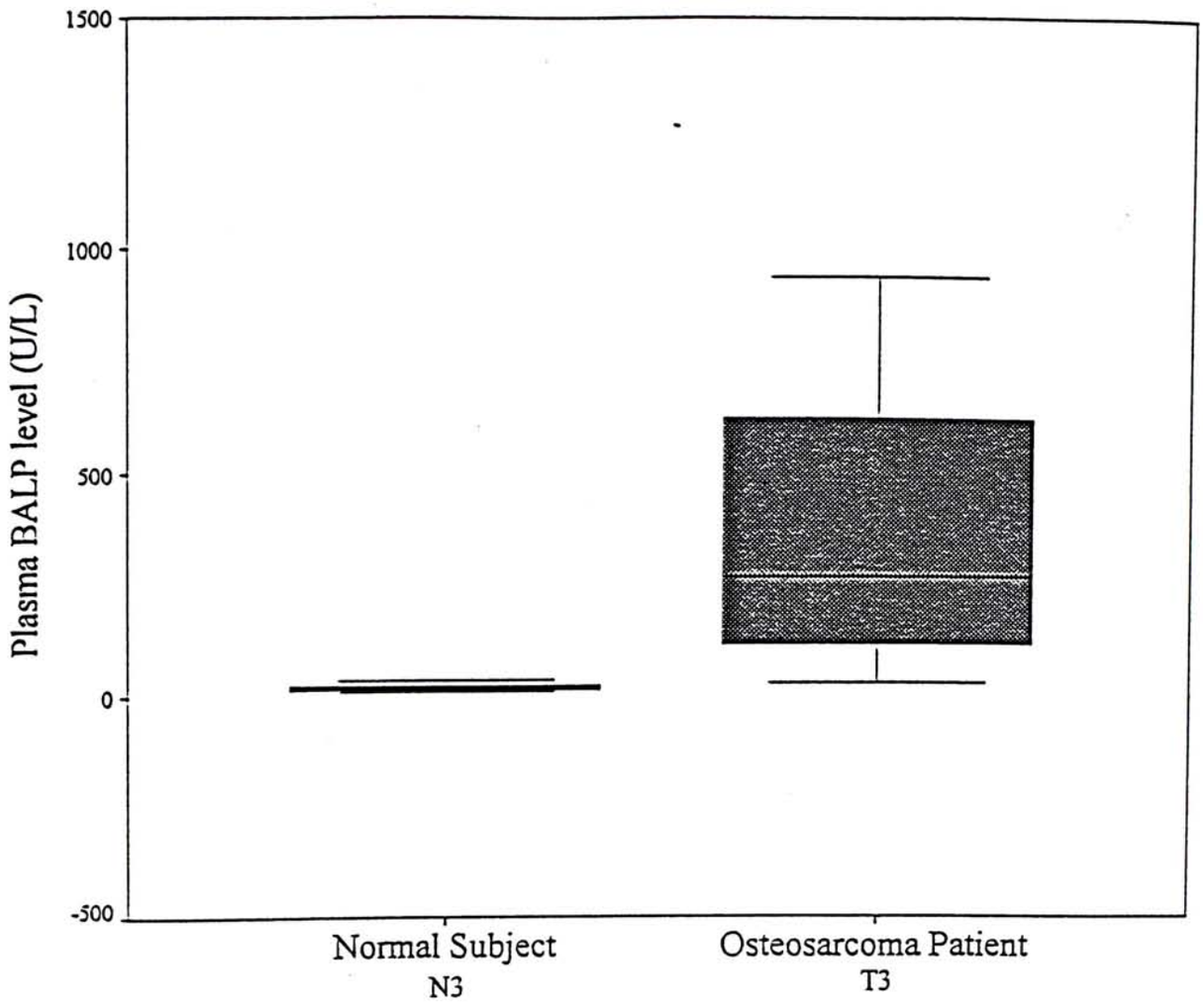


Figure 3.8. Boxplot of Plasma BALP level in Normal Subjects (N3) and Osteosarcoma Patients (T3) with age above 16.
For osteosarcoma patients, the plasma BALP level is the BALP-Adm Analysis done by Student's t-test, with $p < 0.001$, between the difference of normals subjects N3 ($n = 103$) and osteosarcoma patients T3 ($n = 26$).

Patient Code	Sex/Age	Group	Plasma TALP-adm (U/L)
CKL	F/9	T1	172.87
KCY	M/10	T1	520.43
LDK	F/11	T1	136.8
TI	M/11	T1	402.85
SKW	M/11	T1	592.42
BWY	F/12	T2	122.71
LYM	F/12	T2	163.35
MLK	F/12	T2	128.85
HWH	M/12	T2	201.97
WCK	M/12	T2	650.98
LWS	M/13	T2	608.28
YCY	M/13	T2	183.84
KSW	F/14	T2	1595.88
LKH	M/14	T2	370.34
CLY	M/14	T2	394.75
CSL	M/15	T2	348.14
PTM	M/16	T3	397.19
CHW	M/17	T3	149.35
LCK	M/17	T3	45.92
SHL	M/17	T3	77.7
CHL	M/18	T3	321.54
KSP	F/18	T3	226.36
AKM	M/18	T3	1093.91
TCY	F/18	T3	267.85
TCC	M/20	T3	208.06
MSK	M/23	T3	347.16
SC	M/24	T3	292.25
HSF	F/26	T3	159.11
CY	F/30	T3	187.32
LMC	M/30	T3	271.51
LW	M/13	T3	193.42
TYC	M/38	T3	376.45
LFH	M/40	T3	186.10
WCW	M/45	T3	335.28
HHW	M/58	T3	56.69

Table 3.8 Plasma Total ALP level at admission of patients

Patients is divided into 3 group according to their age at diagnosis. T1= age below 12; T2 = age between 12 to 16 and; T3 = age above 16.

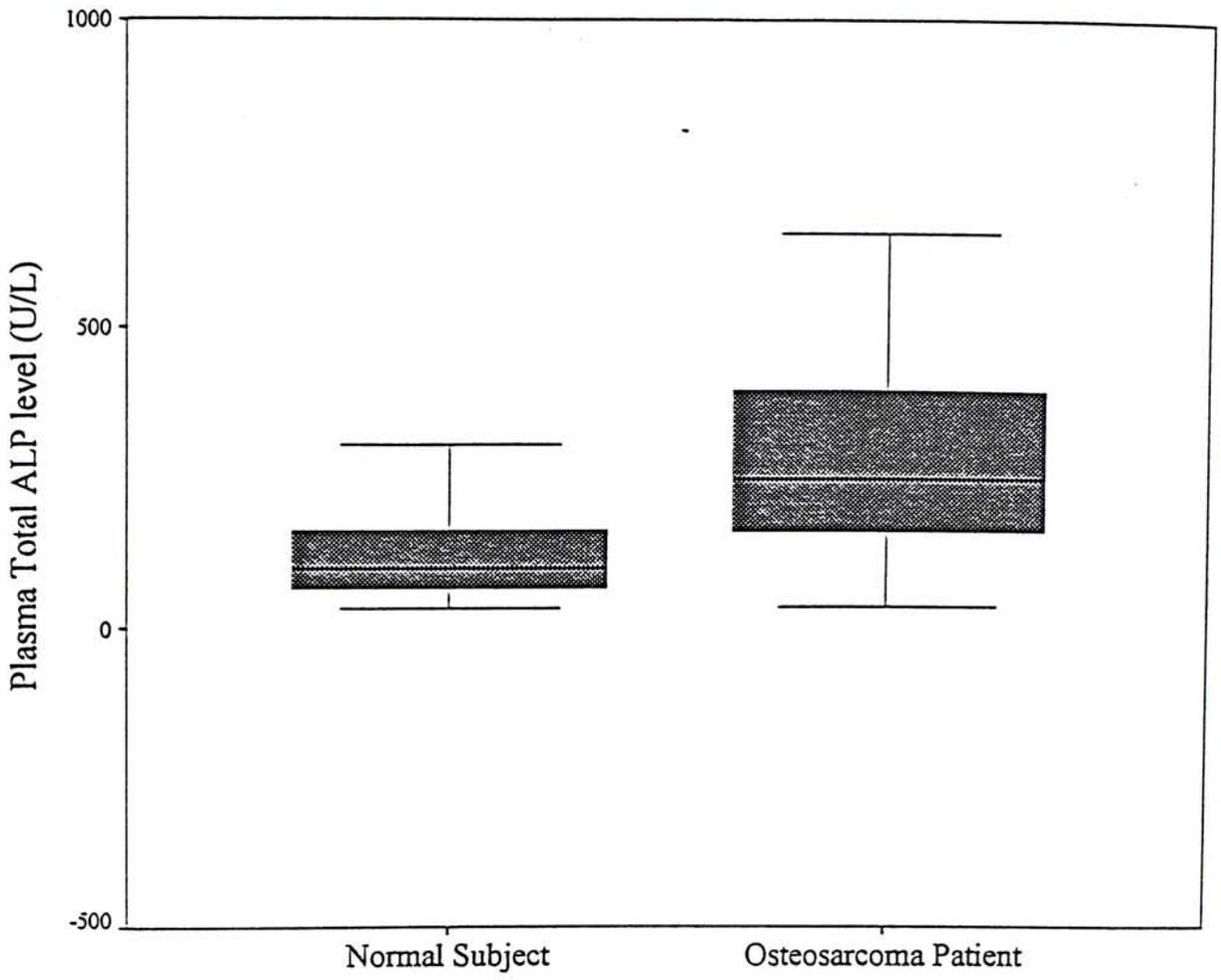


Figure 3.9 Boxplot of plasma Total ALP level in Normal Subjects and in Osteosarcoma Patients, independent of ages.
Analysis done by 2-Way ANOVA, $p < 0.001$, between difference of normal subjects (n = 136) and osteosarcoma patients (n = 38).

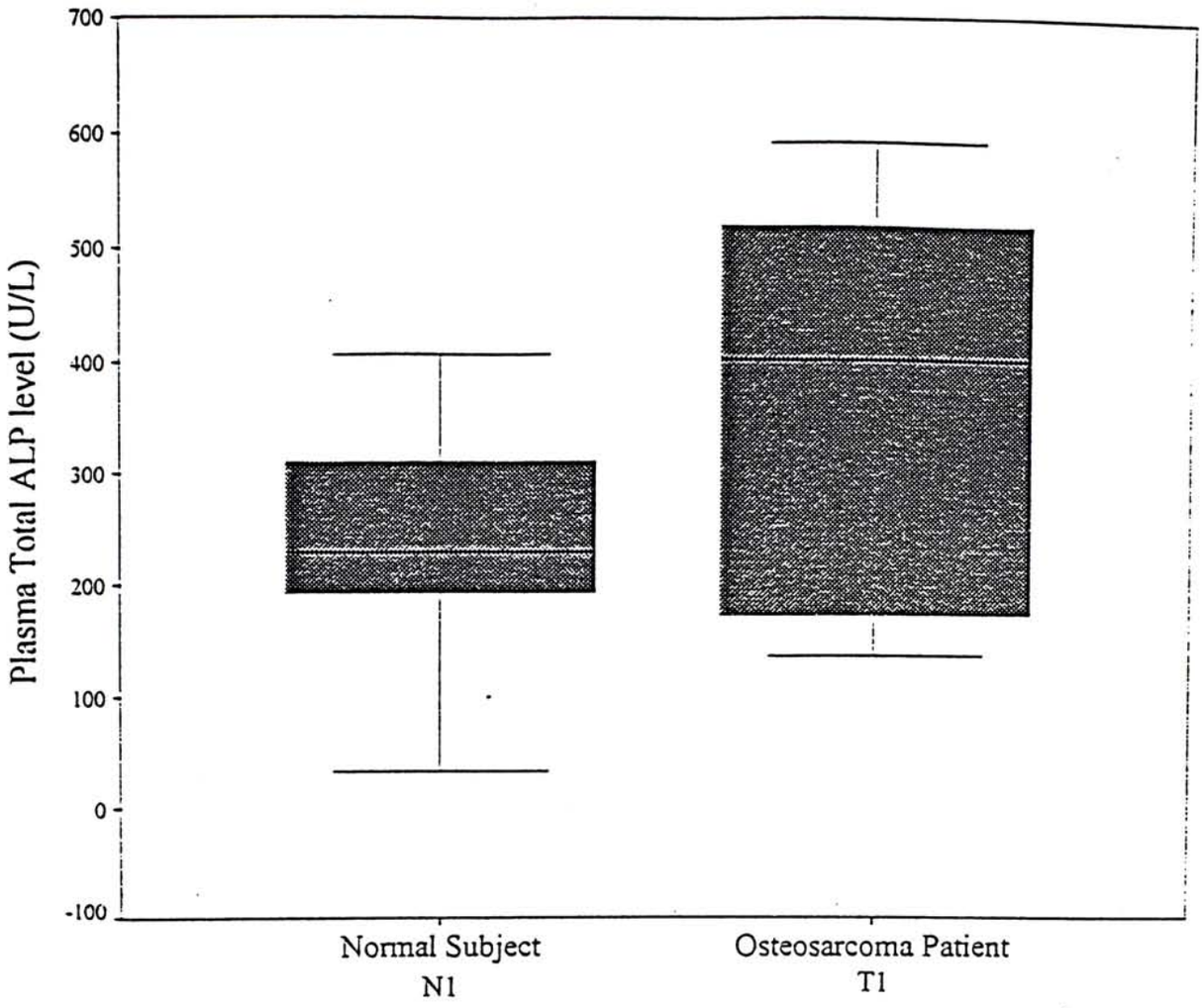


Figure 3.10. Boxplot of Plasma Total ALP level in Normal Subjects (N1) and Osteosarcoma Patients (T1) with ages below 12.

For osteosarcoma patients, the Plasma Total ALP level is the TALP-Adm

Analysis done by Student's t-test, with $p > 0.1$, between normal subjects N1 ($n = 25$) and osteosarcoma patients T1 ($n = 5$).

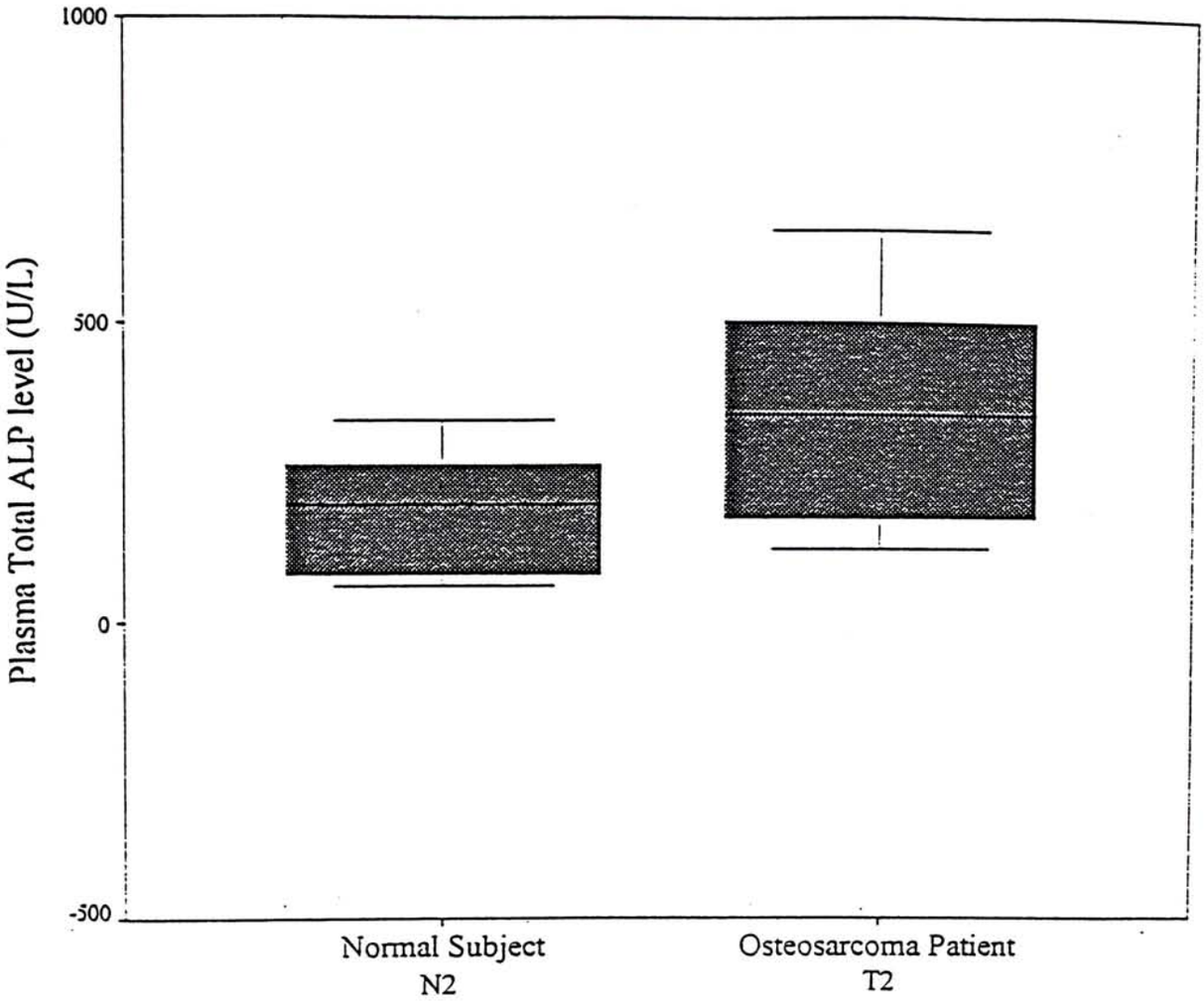


Figure 3.11. Boxplot of Plasma Total ALP level in Normal Subjects (N2) and Osteosarcoma Patients (T2) with ages betweeen 12-16.

For osteosarcoma patients, the Plasma Total ALP level is the TALP-Adm

Analysis done by Student's t-test, with $p > 0.05$, between different of normal subjects N2 ($n = 10$) and osteosarcoma patients T2 ($n = 11$).

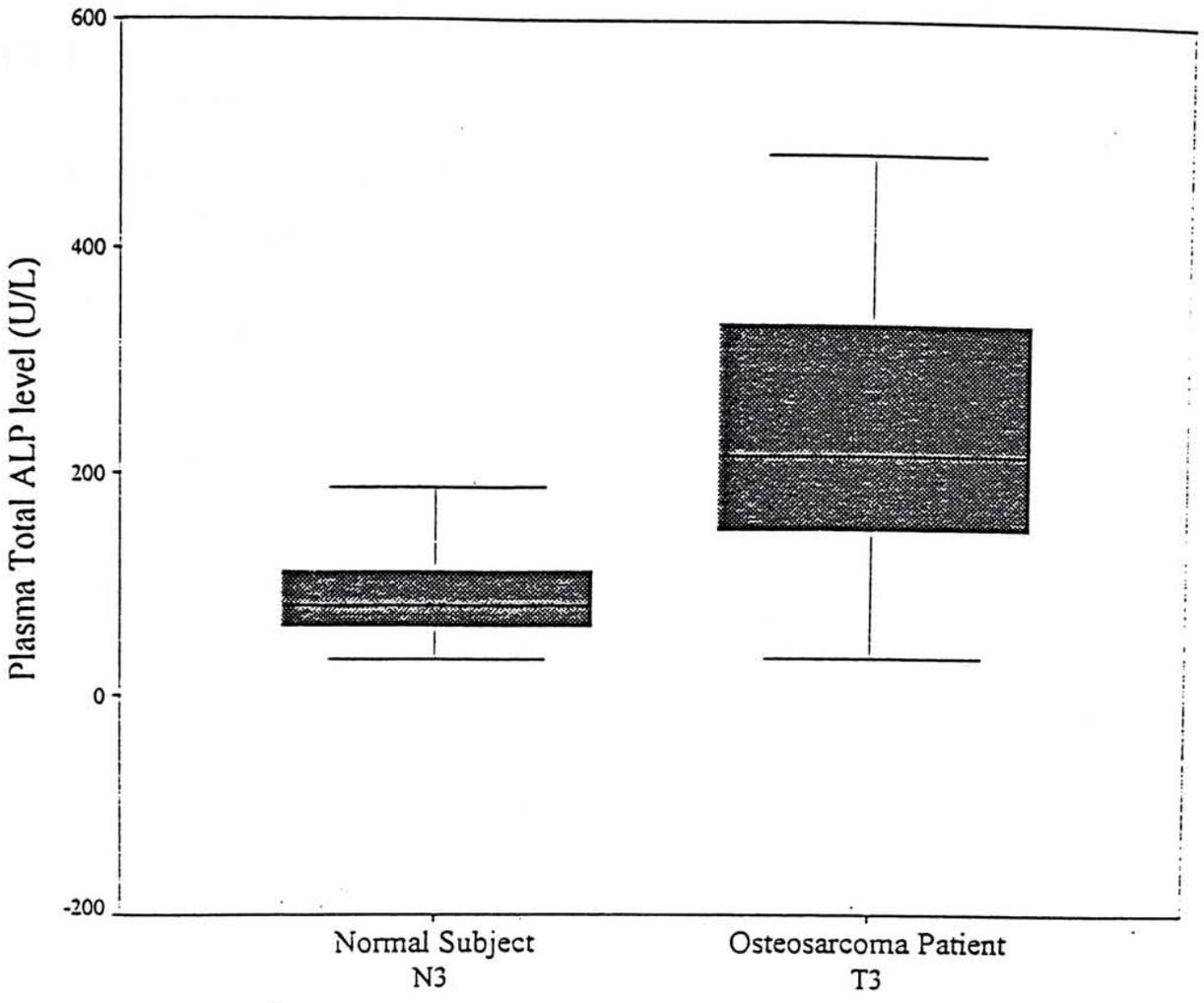


Figure 3.12. Boxplot of Plasma Total ALP level in Normal Subjects (N3) and Osteosarcoma Patients (T3) with ages above 16.

For osteosarcoma patients, the Plasma Total ALP level is the TALP-Adm

Analysis done by Student's t-test, with $p < 0.005$, between difference of normal subjects N3 ($n = 101$) and osteosarcoma patients T3 ($n = 22$).

significantly higher ($p < 0.001$) compared with the patients who did not have local recurrence (Figure 3.13).

3.2.4.b Correlation of plasma BALP-Adm with survival rate of the patients

3.2.4.b.i One year Survival Rate

Plasma BALP-Adm of patients with at least 1-year follow-up period was listed in Table 3.10. Five out of 33 patients died from the disease within one year of studies. Among these 5 patients, 4 of them received operative removal of the osteosarcoma and one of them died before the surgery. Figure 3.14 shows that the plasma BALP-Adm level is significantly higher compared with those patients who died from the disease within one year than patients who survived ($p = 0.001$).

3.2.4.b.ii Two-year Survival Rate

Patients with 2 years follow-up periods and their BALP-Adm are shown in Table 3.11. 35.5% of the patients died of the disease mainly due to local recurrence and metastasis of the tumor. When compared with BALP-Adm of patients who survived, patients who died within 2 years have a significantly higher BALP-Adm ($p = 0.001$). Figure 3.15 demonstrates the difference in plasma BALP-Adm in these two groups.

3.2.4.b.iii Three year survival rate

Patients with 3-year follow-up periods and their BALP-Adm are shown in Table 3.12. Only 53.3 % of patients survived three years or more. Again, plasma BALP-Adm of patients who died within 3 year studies is significant higher ($p = 0.05$) than the patients who survived. Results are shown in Figure 3.16.

3.2.4.c Correlation of the plasma BALP-Adm with the tumor volume

The Diagnostic Radiology & Organ Imaging Department, Prince of Wales Hospital calculated tumor volume of the patients. This value is

estimated from the Magnetic Resonance Imaging (MRI) of the patients using Ellipsoid Approximation. Patients' tumor volume and their plasma BALP-Adm are shown in Table 3.13. We find that there is a significant linear correlation ($p = 0.045$) between the BALP-Adm and the tumor volume (Figure 3.17). The correlation found is 0.6127 with $R^2 = 0.3753$.

3.2.5 Using Plasma BALP measurement for monitoring of the disease.

3.2.5.a Effectiveness of Pre-operative Chemotherapy

The effectiveness of the pre-operative chemotherapy is reflected by the tumor necrosis of the resected tumor specimen. After the operative removal of the tumor, the specimen is subject to histological studies to determine how much of the tumor is killed by the pre-operative chemotherapy. The Anatomical and Cellular Pathology Department, Prince of Wales Hospital determined the degree of tumor necrosis, i.e. percentage of tumor cells killed. The tumor necrosis of the patients receiving pre-operative chemotherapy and excision of the tumor is presented in Table 3.14.

Patients' plasma BALP level after the pre-operative chemotherapy was compared with the age-matched normal reference values. Patients whose plasma BALP levels returned to the age-matched normal range after the chemotherapy are classified as "DROP". Patients with their plasma BALP level higher than the age-matched normal range even after the chemotherapy are classified as "REMAIN".

Independent sample Student's t-test analysis shows that, the degree of tumor necrosis in the "DROP" group is significantly higher than whose patients belong to the "REMAIN" group, with two-tailed significance of 0.021 (Figure 3.18).

3.2.5.b Change of Plasma BALP level during the treatment

3.2.5.b.i Monitoring of pre-operative chemotherapy

In order to monitor the change of plasma BALP activity in the osteosarcoma patients during treatment, blood samples were collected at different time intervals. (i) at admission; (ii) during the preoperative chemotherapy; (iii) day before and after the resection of the tumor; (v) during postoperative chemotherapy and (vi) at every follow-up clinic. Blood samples were also collected during (vii) relapse or (viii) secondary metastasis.

Patients were divided into 2 groups according to their response to chemotherapy. Classification is based on (1) clinical observation if patients have not had their tumor resected or (2) by the degree of patients' tumor necrosis following tumor resection. Patients with degree of tumor necrosis higher than 80% were considered as good responders to chemotherapy while those were lower than 50% were considered as poor responder.

Figure 3.19 shows the change of plasma BALP activity during the pre-operative chemotherapy of patients that's responded well toward the chemotherapy. There is a general trend that the plasma BALP levels dropped during the chemotherapy in whose patients with initial high values. Most of the patients have their plasma BALP drop back to normal range before the operation.

The change of plasma BALP levels in whose patients with poor response toward the chemotherapy was shown in Figure 3.20. Compared with the patients with good response, the plasma BALP levels of patients with poor response did not show significant drop during the chemotherapy. Large fluctuation of the plasma BALP was observed in three of the patients. One patient (KSW) died before the operation and the other 2 patients (TI and SC) also died within half year of operation. Moreover, the plasma BALP levels did not return to normal even after the removal of the tumor.

Patient Code	Sex/Age	Plasma BALP-Adm (U/L)	Date of Recurrence
TI	M/10	244.84	5 MONTH
PTM	M/16	789.23	9 MONTH
CHW	M/17	168.72	18 MONTH
CML	F/18	702.77	24 MONTH
YKC	M/18	837.10	6 MONTH
MCW	M/21	790.01	2 MONTH
SC	M/24	934.37	4 MONTH
CKL	F/9	192.58	
KCY	M/10	220.80	
WL	F/11	99.40	
YSM	M/11	566.13	
SKW	M/11	271.88	
YLP	F/12	521.35	
BWY	F/12	102.80	
LYM	F/12	107.66	
MLK	F/12	97.22	
WCK	M/12	290.60	
TCW	M/12	215.69	
LWS	M/13	631.75	
YCY	M/13	64.64	
LKH	M/14	326.03	
CLY	M/14	178.80	
LCK	M/17	34.54	
SHL	M/17	71.68	
CCK	F/18	691.96	
TCY	F/18	119.78	
KSP	F/18	87.99	
CHL	M/18	319.09	
AKM	M/18	494.92	
TCC	M/20	81.75	
AMW	M/21	289.75	
PKwok	F/22	527.53	
MSK	M/23	547.60	
HSF	F/26	215.38	
CY	F/30	624.03	
LMC	M/30	115.80	
LW	F/36	67.56	
TYC	M/38	158.37	
LFH	M/40	245.75	
WCW	M/45	228.65	
HHW	M/58	28.97	

Table 3.9. Plasma BALP level at admission of patients with / without local recurrence.

All patient with complete post-operative chemotherapy done.
Date of the recurrence were counted from date of diagnosis

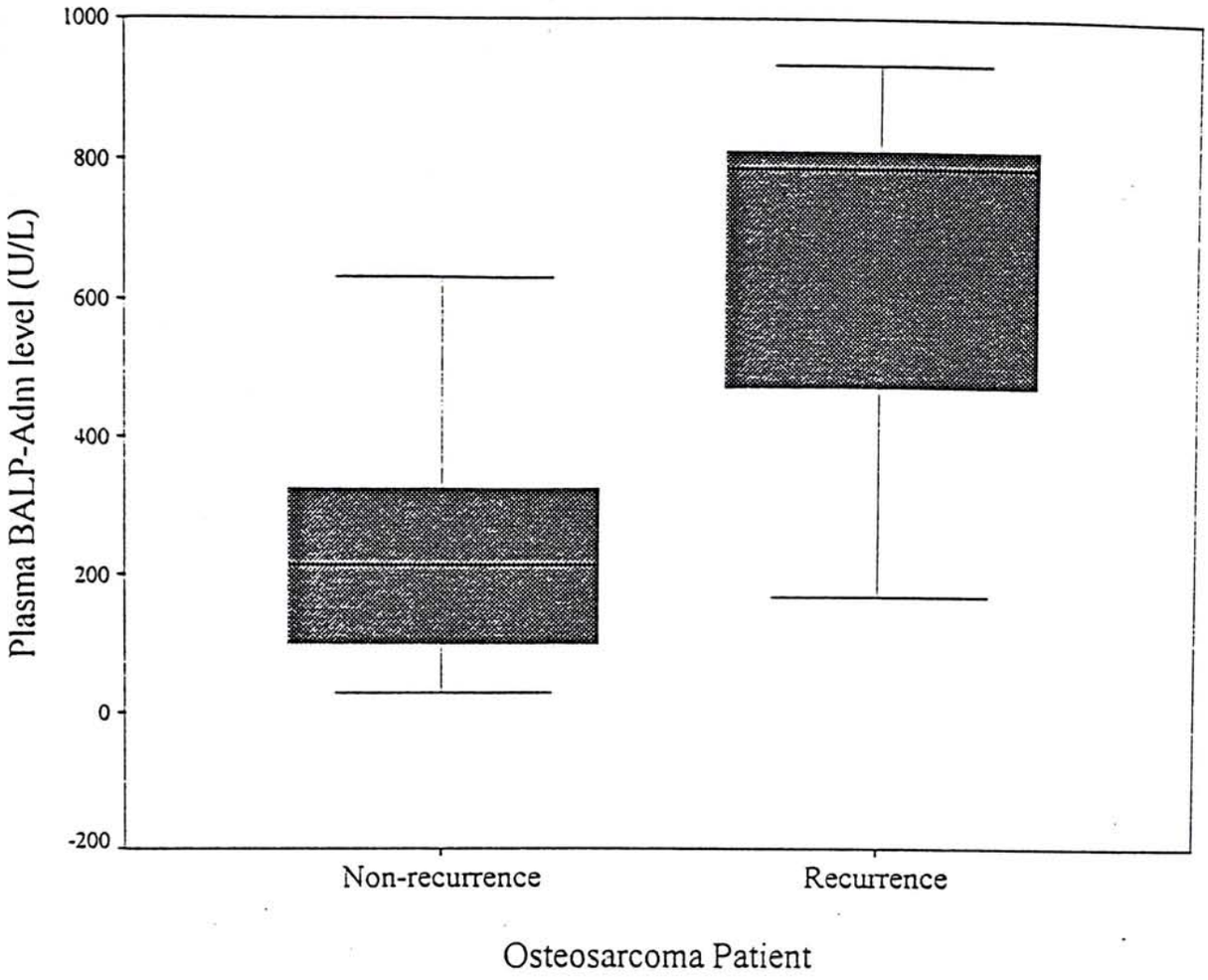


Figure 3.13. Boxplot of Plasma BALP-Adm level in Osteosarcoma Patients with or without Local Recurrence.

Analysis is done by Student's t-test, $p < 0.001$, between the difference of non-recurrent patients ($n = 34$) and the recurrence patients ($n = 7$).

Patient Code	Sex/Age	Die of disease within 1 year	Plasma BALP-Adm (U/L)
TI	M/10	11 month	244.84
YKC	M/18	11 month	837.10
KSW	F/14	5 month	1057.49
MCW	M/21	9 month	790.01
SC	M/24	9 month	934.37
KCY	M/10		220.80
YSM	M/11		566.13
SKW	M/11		271.88
YLP	F/12		521.35
WCK	M/12		290.60
TCWJ	M/12		215.69
BWY	F/12		102.80
LYM	F/12		107.66
LWS	M/13		631.75
LKH	M/14		326.03
CLY	M/14		178.80
PTM	M/16		789.23
CHW	M/17		168.72
LCK	M/17		34.54
CHL	M/18		319.09
CML	F/18		702.77
CCK	F/18		691.96
AKM	M/18		494.92
TCY	F/18		119.78
KSP	F/18		87.99
TCC	M/20		81.75
AMW	M/21		289.75
KP	F/22		527.53
MSK	M/23		547.60
CY	F/30		624.03
LMC	M/30		115.80
LW	F/36		67.56
TYC	M/38		158.37
LFH	M/40		245.75

Table 3.10. Plasma BALP-Adm of patients with at least one year follow-up period and their survival.

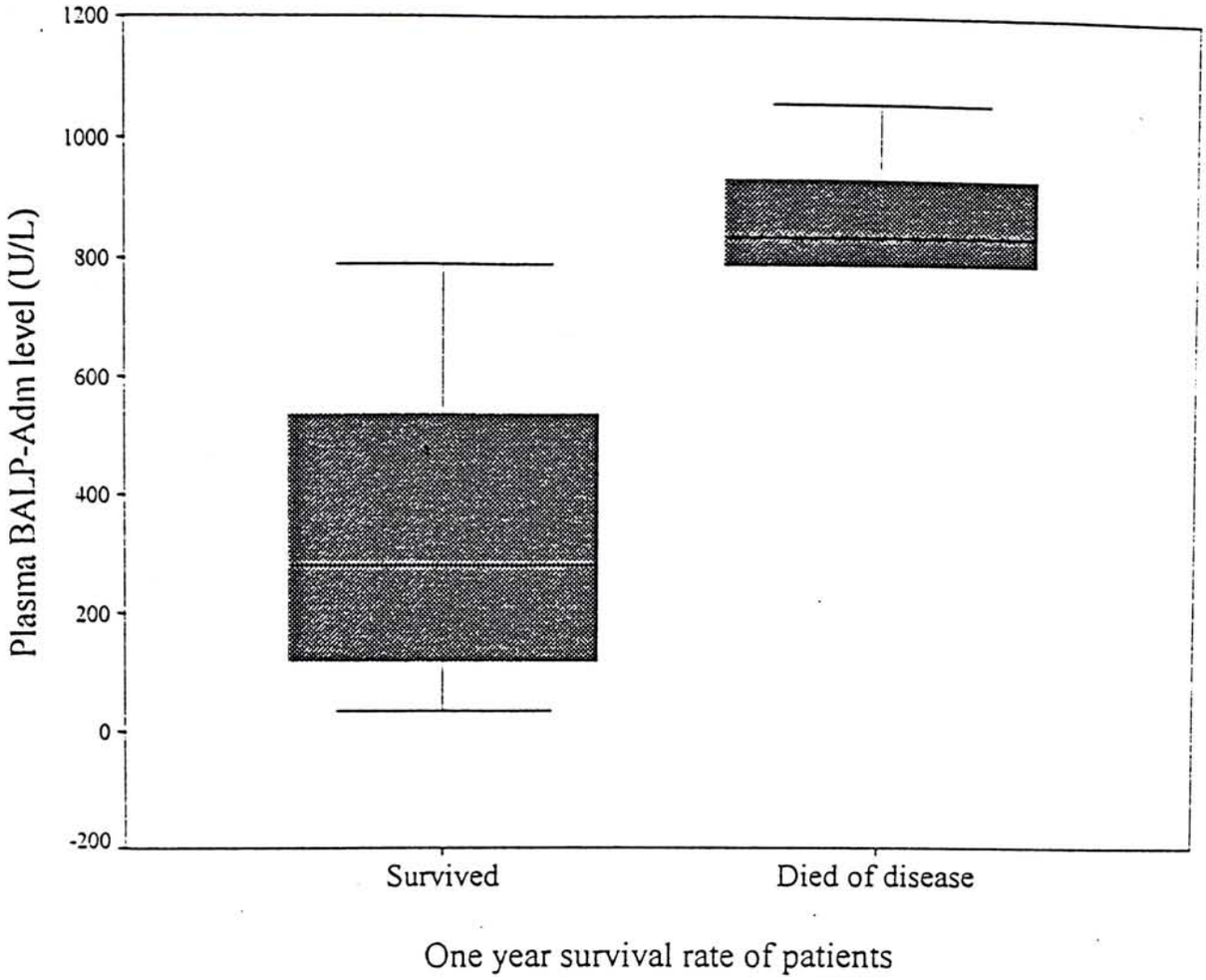


Figure 3.14 Boxplot of Plasma BALP at admission in osteosarcoma patients with one-year survival or died of disease within one year.

Analysis is done by Student's t-test, p = 0.001, between the difference of survivied patients (n = 28) and patients who died of disease (n = 5).

Patient Code	Sex/Age	Die of disease within 2 years	Plasma BALP-Adm (U/L)
KSW	F/14	5 months	1057.49
MCW	M/21	9 months	790.01
SC	M/24	9 months	934.37
TI	M/10	11 months	244.84
YKC	M/18	11 months	837.10
AKM	M/18	13 months	494.92
CHL	M/18	16 months	319.09
KP	F/22	19 months	527.53
TCWJ	M/12	24 months	215.69
PTM	M/16	24 months	789.23
CCK	F/18	24 months	691.96
KCY	M/10		220.80
YSM	M/11		566.13
SKW	M/11		271.88
YLP	F/12		521.35
WCK	M/12		290.60
LWS	M/13		631.75
LKH	M/14		326.03
CLY	M/14		178.80
LCK	M/17		34 54
CML	F/18		702.77
TCY	F/18		119.78
KSP	F/18		87.99
TCC	M/20		81.75
AMW	M/21		289.75
MSK	M/23		547.60
CY	F/30		624.03
LMC	M/30		115.80
LW	F/36		67.56
TYC	M/38		158.37
LFH	M/40		245.75

Table 3.11. Plasma BALP-Adm of patients with at least two year follow-up period and their survival.

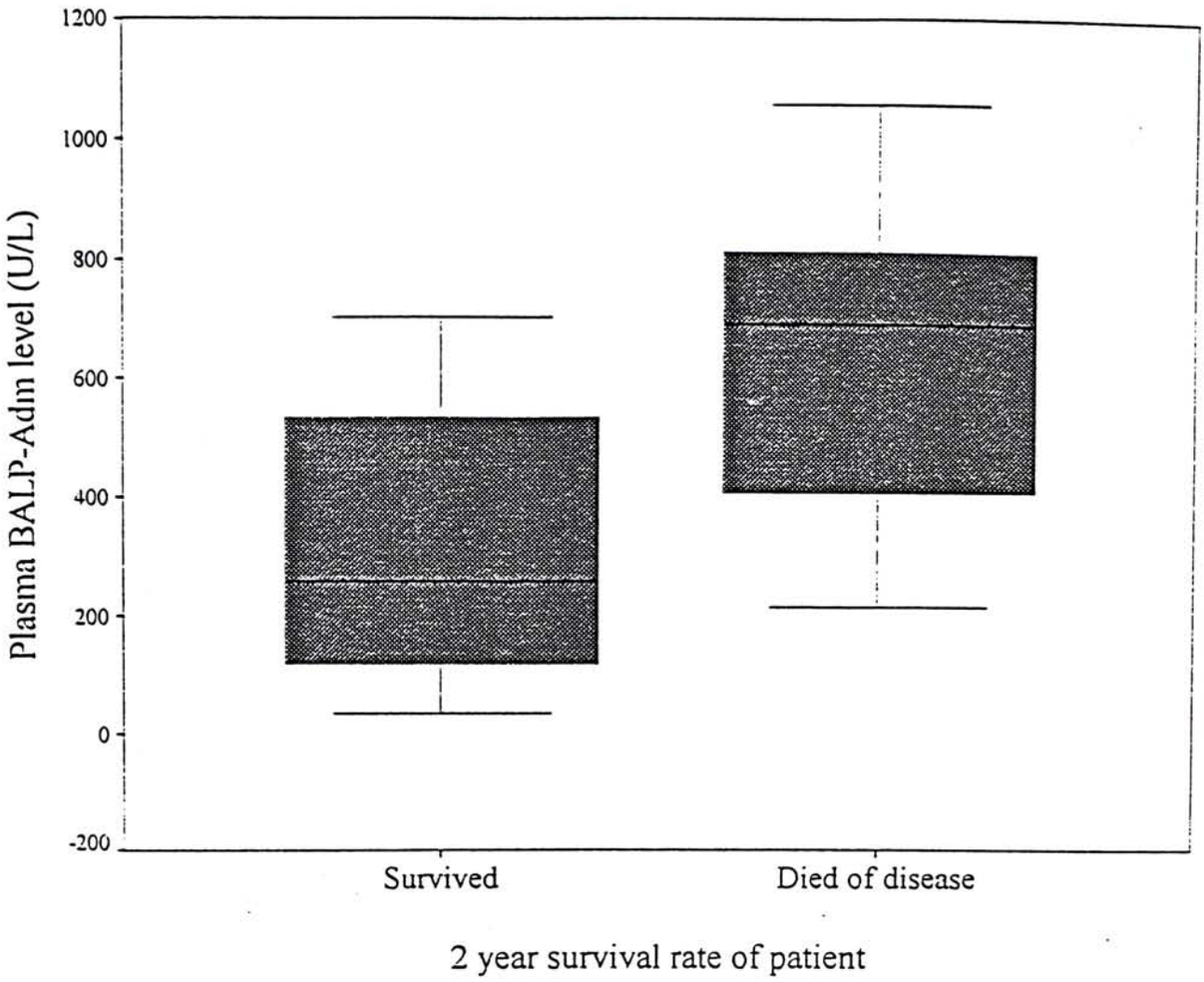


Figure 3.15 Boxplot of Plasma BALP at admission in osteosarcoma patients with two-year survival or died of disease within two-years.

Analysis is done by Student's t-test, p = 0.001, between the difference of survived patients (n = 20) and patients who died of disease (n = 11).

Patient Code	Age/Sex	Die of disease within 3 years	Plasma BALP-Adm (U/L)
KSW	F/14	5 months	1057.49
MCW	M/21	9 months	790.01
SC	M/24	9 months	934.37
TI	M/10	11 months	244.84
YKC	M/18	11 months	837.10
AKM	M/18	13 months	494.92
CHL	M/18	16 months	319.09
KP	F/22	19 months	527.53
TCWJ	M/12	24 months	215.69
PTM	M/16	24 months	789.23
CCK	F/18	24 months	691.96
LMC	M/30	26 months	115.80
SKW	M/11	35 months	271.88
LFH	M/40	36 months	245.75
KCY	M/10		220.30
YSM	M/11		566.13
YLP	F/12		521.35
WCK	M/12		290.60
LWS	M/13		631.75
LKH	M/14		326.03
CLY	M/14		178.80
CML	F/18		702.77
TCY	F/18		119.78
KSP	F/18		87.99
TCC	M/20		81.75
AMW	M/21		289.75
MSK	M/23		547.60
CY	F/30		624.03
LW	F/36		67.56
TYC	M/38		158.37

Table 3.12. Plasma BALP-Adm of patients with at least three years follow-up period and their survival.

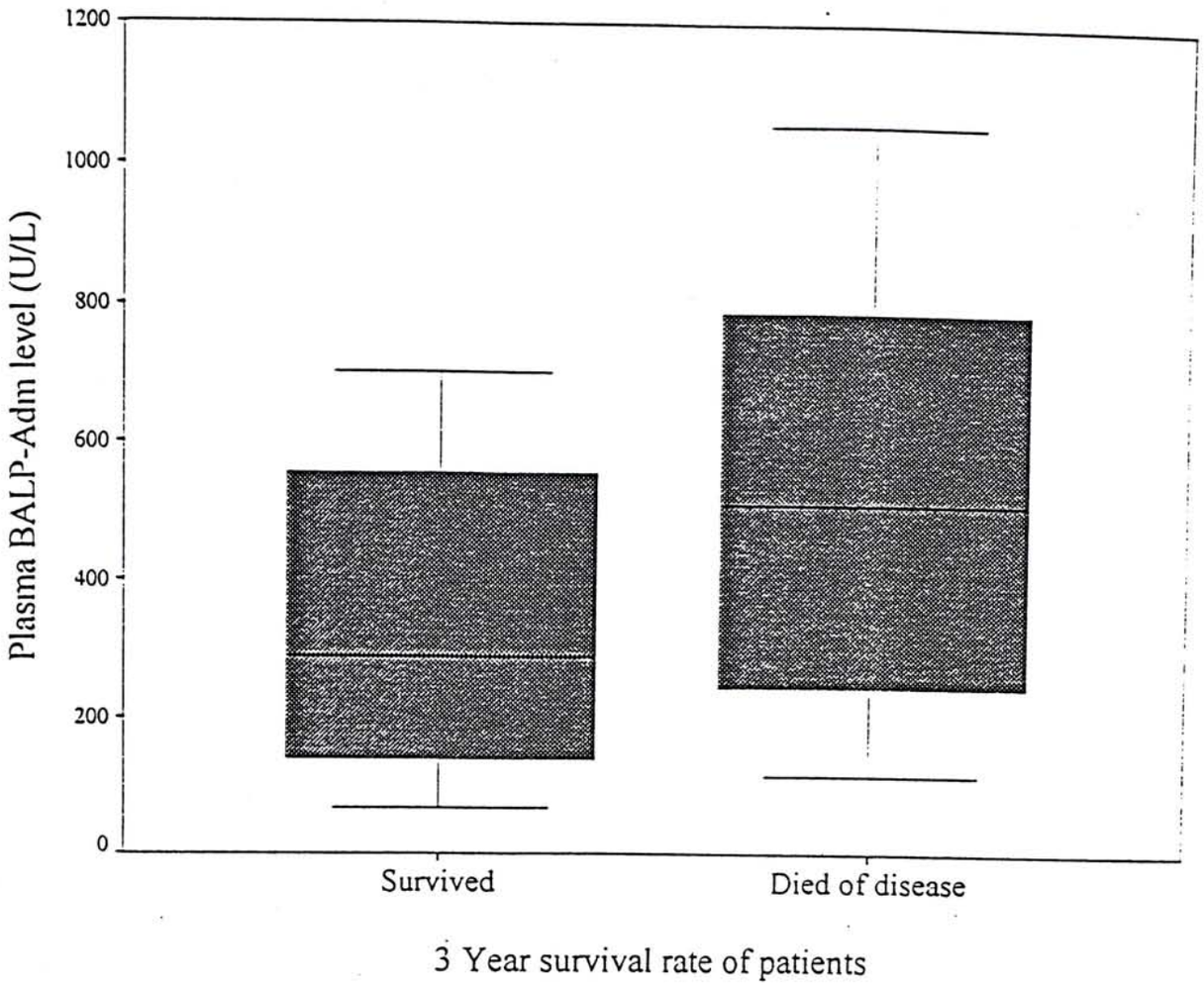


Figure 3.16 Boxplot of Plasma BALP at admission in osteosarcoma patients with three-year survival or died of disease within three years.

Analysis is done by Student's t-test, p = 0.05, between the difference of survived patients (n = 16) and patients who died of disease (n = 14).

Patient Code	Sex/Age	Tumor Volume (ml) *	Plasma BALP-Adm (U/L)
LDK	F/11	280	99.4
BWY	F/12	150	102.80
LYM	F/12	160	107.66
KSW	F/14	300	1057.49
HSF	F/26	280	215.33
TI	M/10	190	244.84
YCY	M/13	135	133.9
CSL	M/15	250	203.26
SHL	M/17	200	71.68
CHW	M/17	300	168.72
MCW	M/21	360	790.01

Table 3.13. Tumor volume and Plasma BALP-Adm of osteosarcoma patients

* Tumour Volume is estimated from the MRI image of the patients using Ellipsoid Approximation.

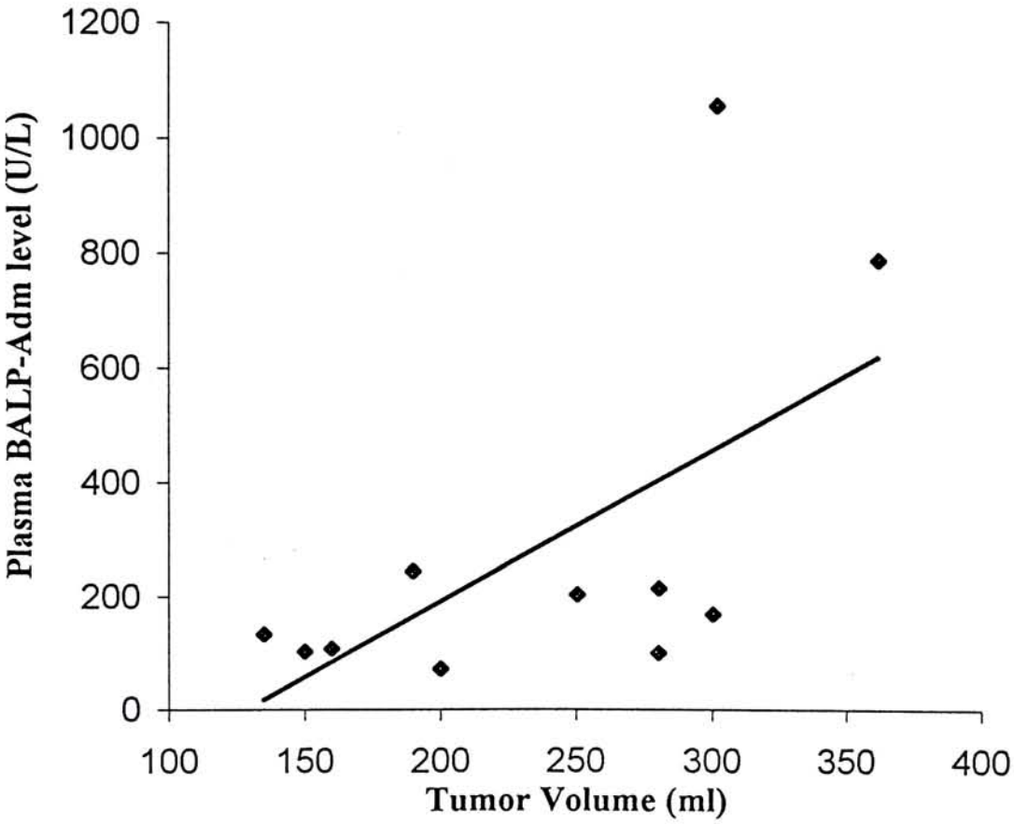


Figure 3.17. Correlation of Plasma BALP at admission and the tumor volume of the osteosarcoma patients

Linear Correlation coefficient = 0.3753 with $p < 0.05$

3.2.5.b.ii Detection of local recurrence and secondary metastasis

For patients with local recurrence or metastasis, blood samples were collected at the time of confirmation of the relapse. Figure 3.21. shows the change of plasma BALP levels in patients with local recurrence or metastasis during the preoperative chemotherapy and at follow-up period. All patients have their plasma BALP level elevated during relapse, some patients exhibited elevated plasma BALP even before clinical confirmation of local recurrence (TI & YCY). Patients with sharp raise of BALP at recurrence (MCW, PTM, LFH, SC & TI) also related to a poor outcome, all those patients died within 6 month after the relapse.

3.3 Alkaline Phosphatase Isozyme expression in Osteosarcoma

3.3.1 Isoelectric point (pI) gradient in Isoelectric Focusing (IEF) gel

The pI gradient of the IEF gel was determined by using internal protein pI marker and is shown in Figure 3.22.

3.3.2 ALP Isozyme Standard

Placental ALP and liver-specific ALP were prepared from human placenta and human liver (obtained from postmortem patients). For placental ALP preparation, 24.6 g of fresh placental tissue was used. The percentage yield of placental ALP extracted is 16% and the final placental ALP standard with activity of 3767 U/L, with specific activity of 8189.86 U/L/mg of protein.

For the liver ALP standard preparation, 15 g of human liver tissue was used. The percentage yield of extraction is around 10% with the final activity of 300U/L and specific activity of 685 U/L/mg of protein.

Cord blood plasma was used as the BALP Standard. This has a total ALP activity of 72.87 U/L and BALP activity of 66.82U/L.

3.3.3 Ectopic Expression of ALP in Extracts of Human Osteosarcoma Cell Line: U-2 OS and SaOS-2

3.3.3.a Isoelectric Focusing Separation

ALP isozymes were extracted from 2 human osteosarcoma cell lines, the U-2 OS and SaOS-2, as described in the methodology section. PALP and LALP standards were prepared as described in the previous section, and cord blood was used as BALP standard. All samples and standards were separated on an IEF gel. Figure 3.23 shows the IEF separation of ALP isozymes with various treatments.

Preheating the sample at 65°C for 6 minutes will essentially denature all forms of ALP except the placental and germ cell ALP, which are very thermostable. On the other hand, 56°C heating for 10 min. will preferably denature the BALP activity as it is much more thermoliable than any other forms of ALP.

In U-2 OS extracts (lane 12-14) and the placental ALP standard (lane 10,11), heating of the samples at both 56°C and 65°C did not affect the ALP activity. Moreover, lane 10 to 14 has a similar position with pI value around 4.4.

In SaOS-2 extracts (lane 1-3) and cord blood (lane 4-5), the ALP expressed are thermoliable as the bands totally disappeared after the 56°C and 65°C heating. Also, the position of the band in SaOS-2 extracts is identical to the main band in the cord blood sample with pI value of 3.9.

In the liver standard (lane 7-8), 56°C heat treatment only partially denatured its ALP activity as the lane 8 only shows reduction of intensity. Complete denaturation of the ALP activity was only observed in the 65°C heat treatment method. Moreover, the band position of liver ALP standard is different from the BALP in cord blood and placental ALP standard with pI value around 3.7.

Patient Code	Sex/Age	Group*	Tumor Necrosis
YSM	M/11	REMAIN	100%
WCK	M/12	REMAIN	50%
LWS	M/13	REMAIN	90%
PTM	M/16	REMAIN	50%
CCK	F/18	REMAIN	60%
CHL	M/18	REMAIN	20%
YKC	M/18	REMAIN	40%
AKM	M/18	REMAIN	0%
AMW	M/21	REMAIN	90%
MCW	M/21	REMAIN	0%
KP	F/22	REMAIN	50%
MSK	M/23	REMAIN	80%
SC	M/24	REMAIN	0%
HSF	F/26	REMAIN	53.80%
CY	F/30	REMAIN	100%
LMC	M/30	REMAIN	0%
LFH	M/40	REMAIN	44%
WCW	M/45	REMAIN	16%
LCS	M/51	REMAIN	50%
CKL	F/9	DROP	58.60%
KCY	M/10	DROP	100%
TI	M/10	DROP	50.80%
LDK	F/11	DROP	82.40%
SKW	M/11	DROP	31%
YLP	F/12	DROP	95%
BWY	F/12	DROP	71.75%
LYM	F/12	DROP	95.50%
TCWJ	M/12	DROP	22%
YCY	M/13	DROP	83%
LKH	M/14	DROP	100%
CHW	M/17	DROP	80%
SHL	M/17	DROP	60%
CML	F/18	DROP	100%

Table 3.14. Tumour necrosis of the osteosarcoma patient in the "DROP" and "REMAIN" group.

*"DROP" and "REMAIN' is classified by comparing the plasma BALP level after preoperative chemotherapy with the age matched normal reference (N1 < 217.7 U/L; N2 < 136.8U/L; N3 < 59.5U/L) with "DROP" group lower than the normal and "REMAIN" group higher than the normal.

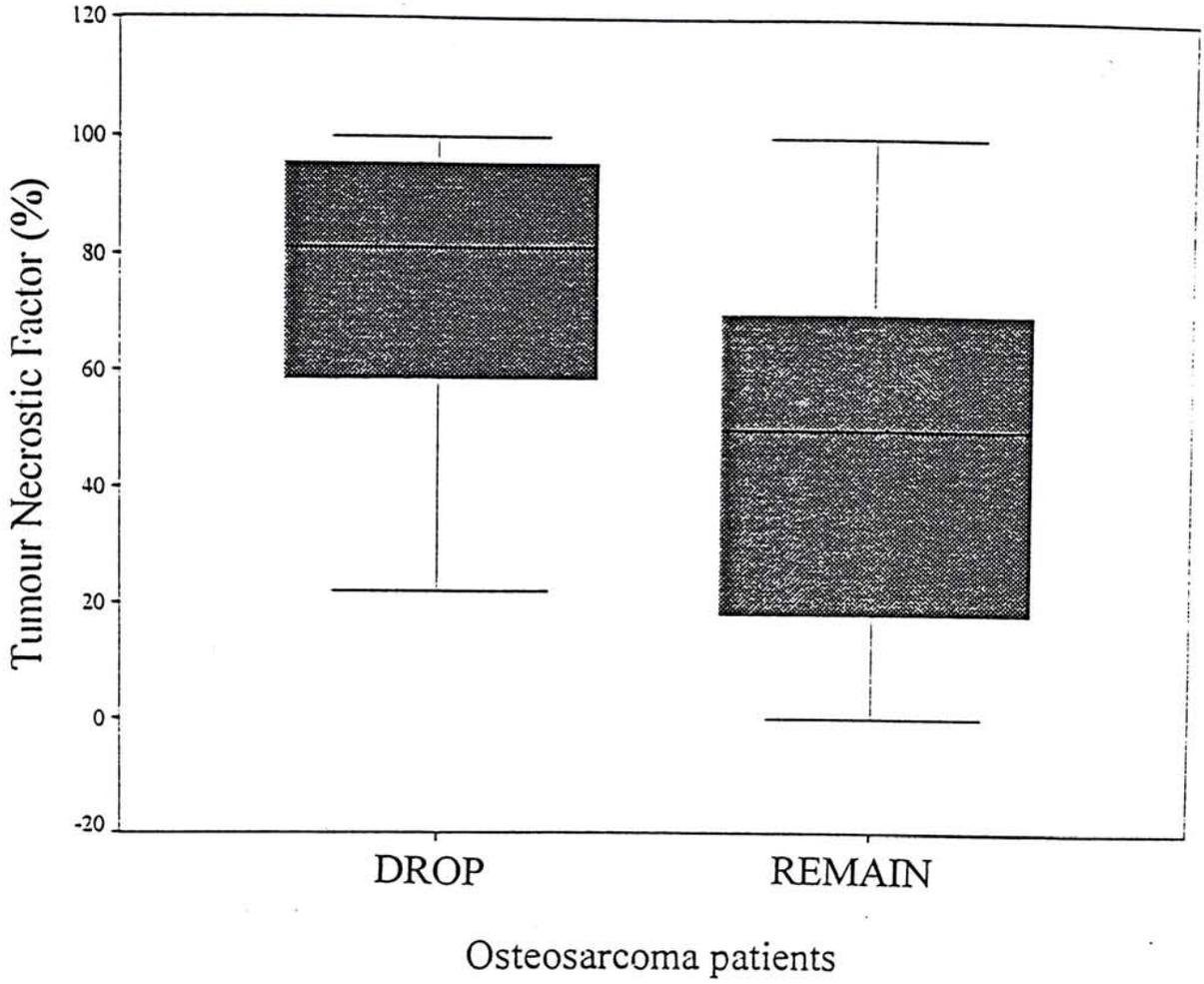


Figure 3.18. Boxplot of degree of tumor necrosis in patients with (DROP group) and without (REAMIN group) of plasma BALP levels during chemotherapy.
Analysis is done by Student's t-test, $p<0.05$, between the difference of "DROP" group (n = 14) and the "REAMIN" group (n = 19).

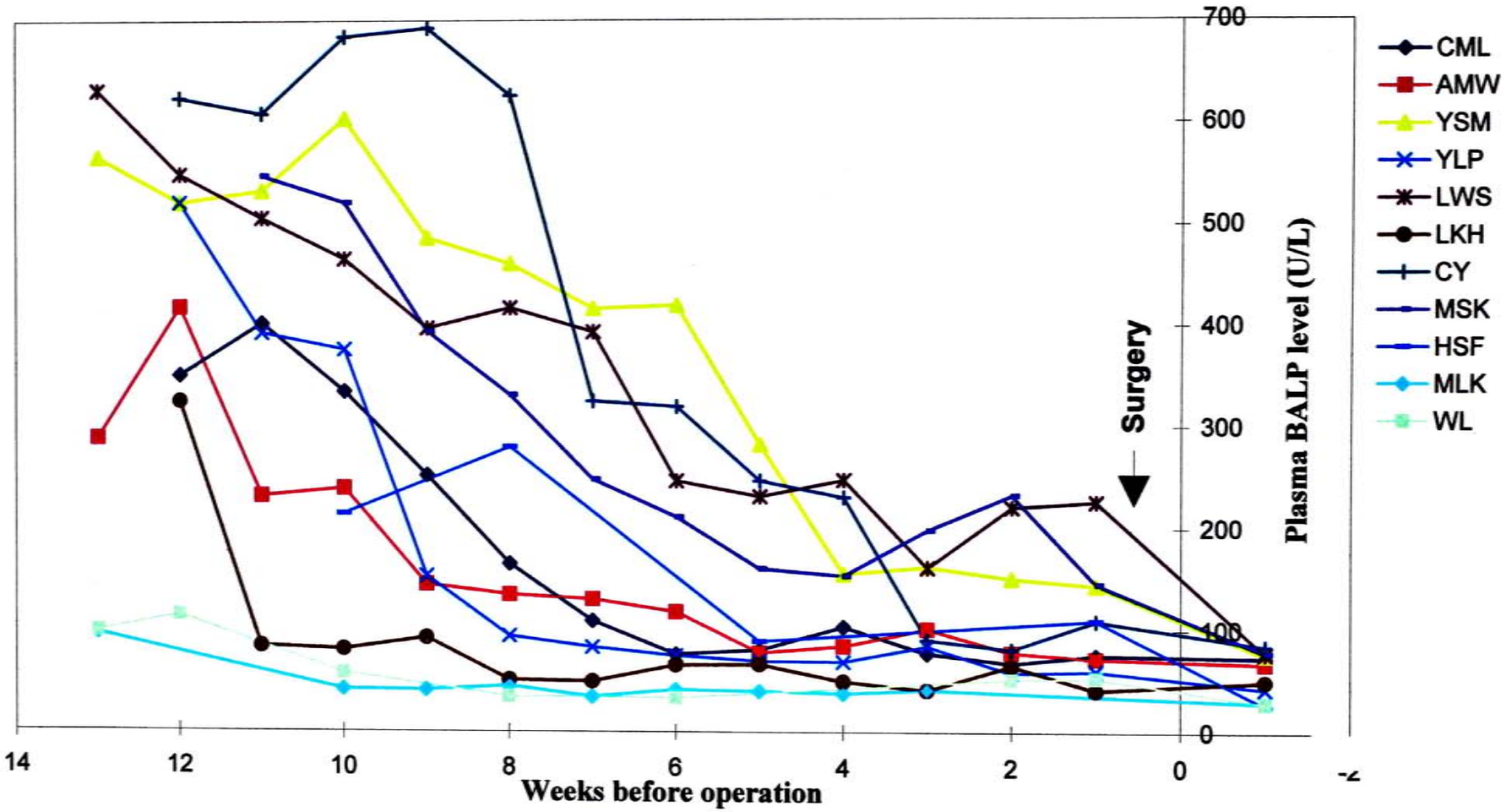


Figure 3.19. Change of Plasma BALP level during pre-operative chemotherapy in patients with good response toward the chemotherapy

Patients are considered as having good response if their tumour necrosis higher than 80%

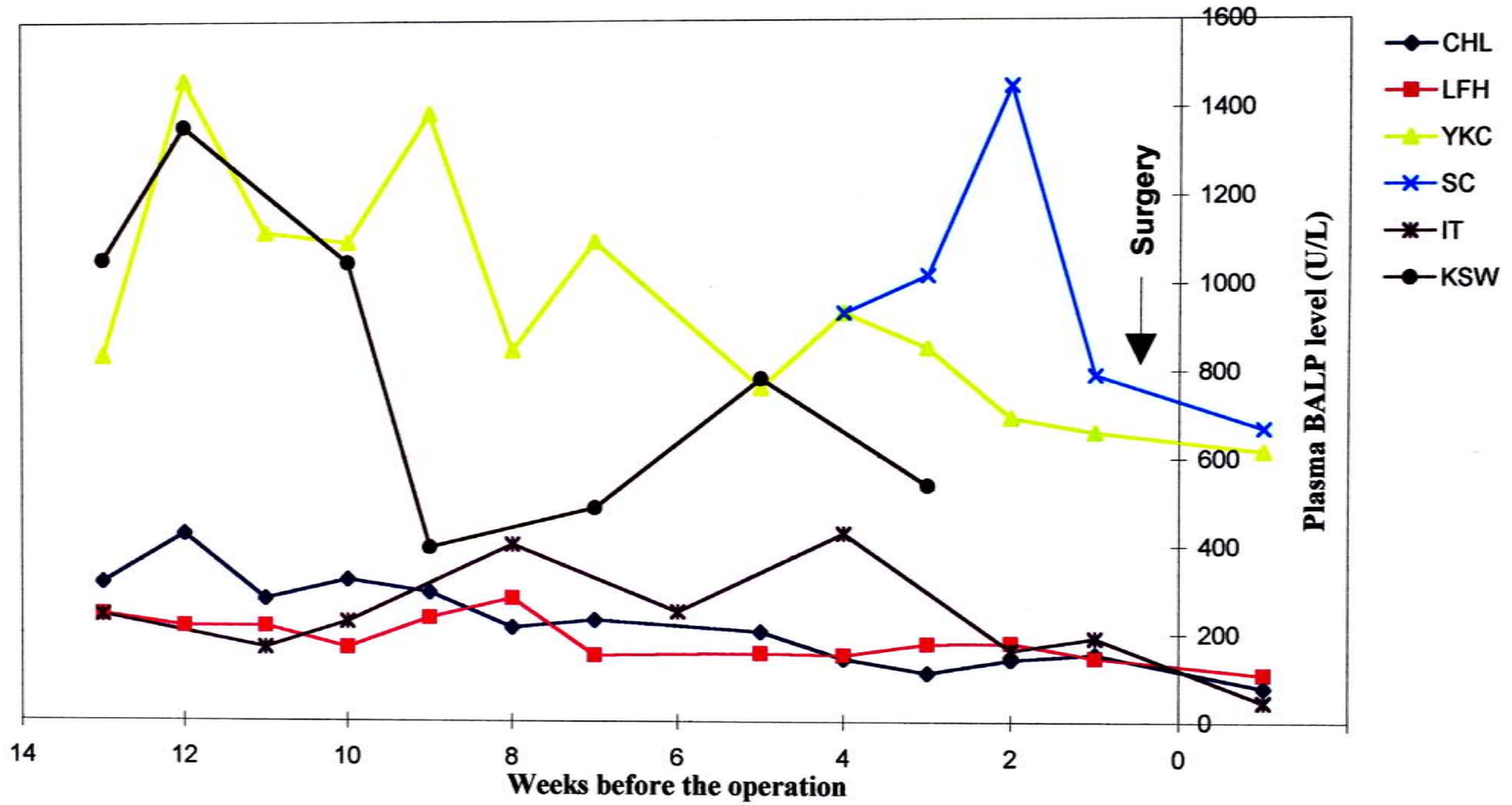


Figure 3.20. Change of Plasma BALP level during preoperative chemotherapy in patients with poor reponse towards chemotherapy

Poor reponse is defined as tumour necrosis lower than 50%

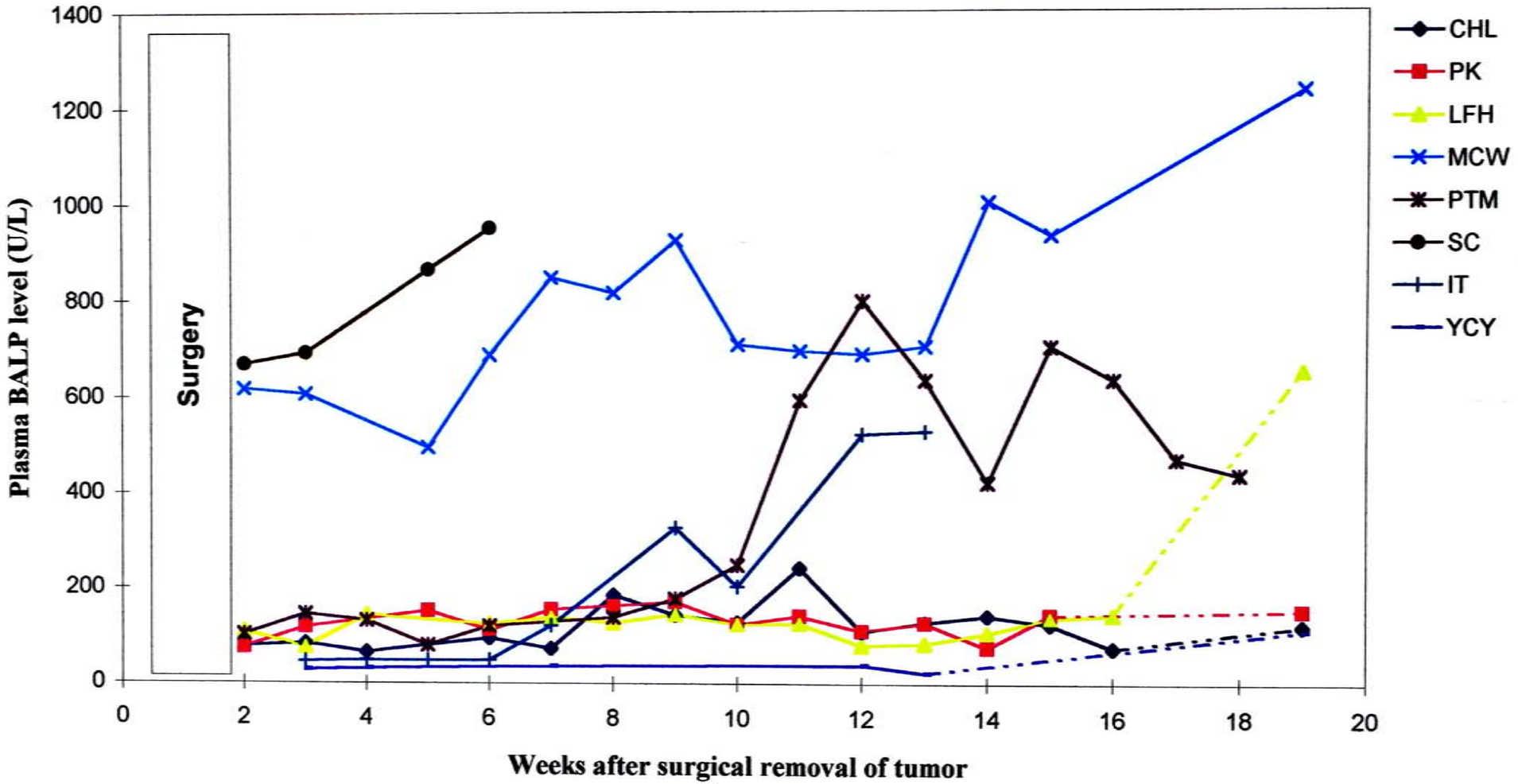


Figure 3.21. Change of Plasma BALP level after surgical removal of tumor in patients with relapse

*Plasma BALP level after 18 week were denoted with dotted line

3.3.3.b Biochemical Differentiation of ALP extracts

Heat inactivation at 56°C and 65°C studies on the ALP extract from the 2 cell lines were compared with the placental ALP standard in Figure 3.24 and Figure 3.25 respectively. ALP expressed in Sa OS-2 cells was inactivated only after a short term of heating, especially with 65°C heating. On the other hand, ALP expressed in U-2 OS cell show only moderate heat inactivation even at 65°C heating, this is similar to the placental ALP standard.

Results of the levamisole and L-phenylalanine inhibition studies were shown in Figure 3.26 and Figure 3.27 respectively. In the levamisole inhibition study, ALP expressed in SaOS-2 is highly sensitive to the inhibition of levamisole with complete inhibition by 1 mM of levamisole. Both placental ALP and ALP expressed in U-2 OS also show inhibition by levamisole, however the degree of inhibition is not as great as Sa-OS 2 ALP extracts. In L-phenylalanine inhibition study, ALP expressed in SaOS-2 is not as sensitive to this inhibitor as in the placental ALP standard (2 mM of 50% inhibition) and U2 OS extracts.

3.3.4 Alkaline Phosphatase Expression in Osteosarcoma Patient Plasma Sample

Plasma sample of the osteosarcoma patients collected at admission was separated by IEF and the isozyme pattern was observed as described in methodology. Figure 3.28 shows the separation of 4 plasma samples.

All plasma samples show 2 bands upon staining with α-naphthyl phosphate coupled with diazonium salt. However, for placental ALP and Bone ALP standard, only one band is observed. In 65°C pre-heating of the samples, all ALP bands disappeared (Figure 3.28B) in the plasma samples. By comparing with the BALP standard, the 2 bands in the plasma sample were identified as BALP and LALP. This is also confirmed by levamisole inhibition studies (Figure 3.28C). With the addition of 0.06mM levamisole into the staining solution, all ALP bands in the plasma samples reduced in intensity even when doubling the incubation time. Moreover lane 11 (BWY)

shows an extra band on prolonged staining with levamisole inhibition. This band compared with the placental ALP standard running on the same gel, exhibited a higher pI value (around 5) and was identified as intestinal ALP.

3.3.5 Alkaline phosphatase isozyme expression in human osteosarcoma biopsy tissue

ALP was extracted from the tissue of a few patients' samples collected during the biopsy was done and IEF gel separation as described in methodology section was done. The expression of ALP in 12 patient's tissue ALP extracts was shown in Figure 3.29. By comparing the PALP, BALP and LALP standard parallel, only BALP isozyme expression was found in human osteosarcoma tissue ALP extracts.

3.3.6 Ectopic Expression of placental ALP in Human Osteosarcoma by Immunohistochemistry

3.3.6.a Ectopic expression of placental ALP in human osteosarcoma cell line U-2 OS

We have demonstrated that there is ectopic expression of PALP in human osteosarcoma cell line. By employing the immunohistochemistry technique with monoclonal antibody against human PALP, positive signal was observed in our seeded U-2 OS cell (Figure 3.30). This result was confirmed by comparing with the negative control (human liver tissue sections) and the positive control (human placenta tissue section) shown in Figure 3.31.

Positive results were represented by the reddish brown precipitation formation. For the human placenta tissue sections, the cells were heavily stained into dark brown (Fig. 3.31A). For the human liver sections, since the primary antibodies have not cross-reactivity with the liver ALP isozyme, the section did not show any brown precipitation (Fig. 3.31B). For the U-2 OS cells, brown precipitation was observed and mainly found in the plasma membrane of the cells, but occasionally some of the cells nucleus were also highlighted (Figure 3.30A).

For the Sa OS-2 cells (Figure 3.32), there was no brown precipitation formation was found in both the slice with (Fig 3.32A) or without (Fig. 3.32B) the primary antibody incubation, thus without the expression of placental ALP isozyme.

3.3.6.b Ectopic expression of placental ALP in human osteosarcoma tissue sections

In our 7 osteosarcoma tissues obtained, they all show negative results in the immunohistostaining, i.e. without the brown precipitation formation, compared with the positive and negative controls. Figure 3.33 & 3.34 shows the H&E staining and the immunohistostaining of PALP in two of the patients' (CSL, WCW) sections.

For CSL, osteosarcoma tissue was obtained in biopsy and the H&E staining (Fig. 3.33A) shown dense population of cancer cells, heavily stained to dark blue with hematoxylene. For immunohistostaining, both the sections with (Fig. 3.33C) or without (Fig. 3.33B) primary antibody incubation, there was not brown color observed and therefore did not shown placental ALP expression.

For WCW, the H&E staining (Fig. 3.34A) shows the dense populated osteosarcoma cells and the lace-like osteoid produced (stained into red) by the cancer cells. Again, the immunohistostaining of patient's tissue sections show no brown color formation and thus no placental ALP expression.

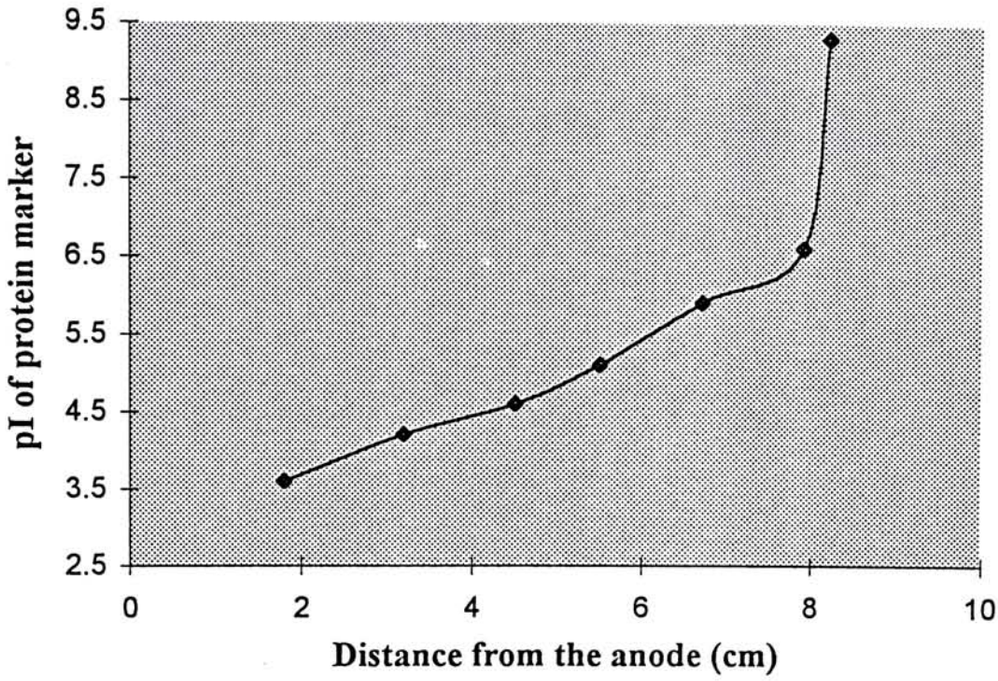


Figure 3.22. pI gradients of Agarose IEF gel

pI of the gel was determined by the protein marker of known pI value and stain with Comassie Blue

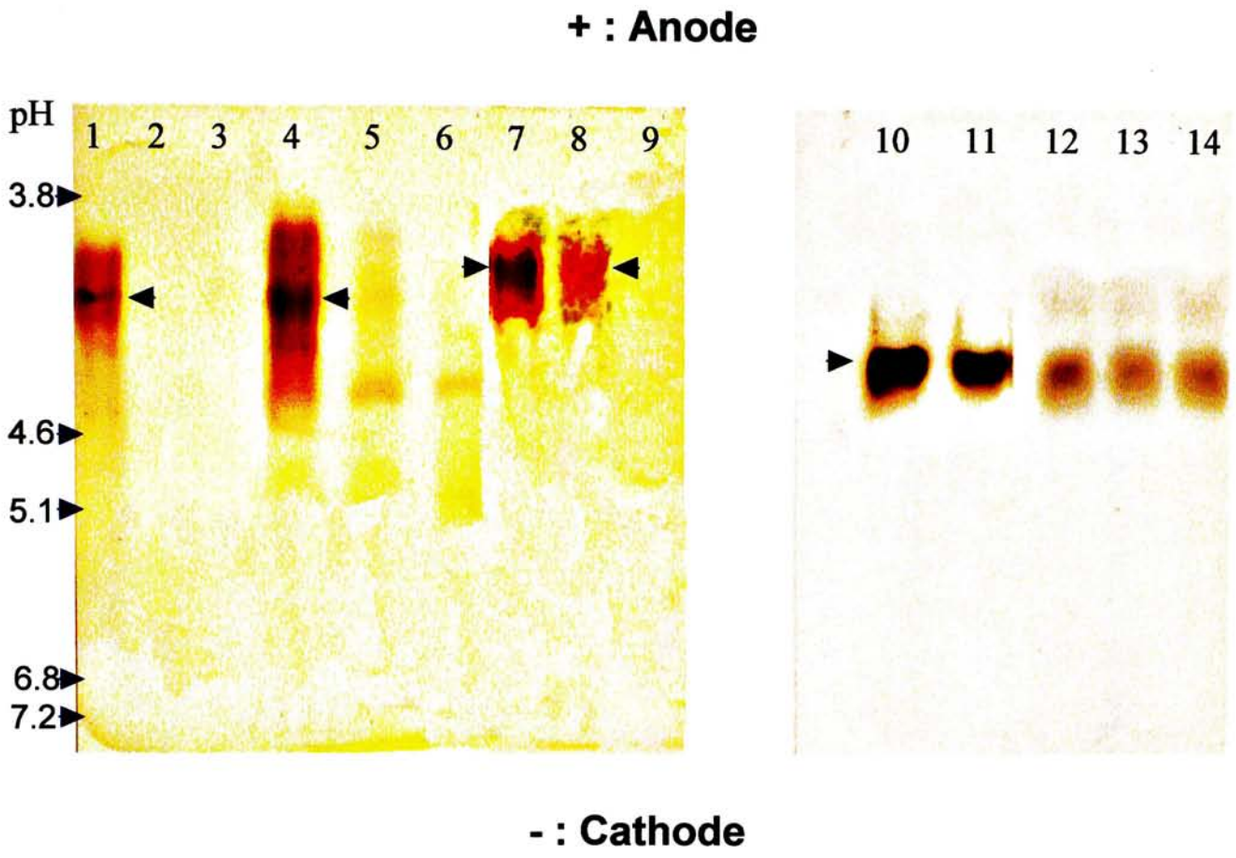


Figure 3.23 IEF separartion of ALP isozymes from various sources.

Lane1,2,3: SaOS-2 extract; Lane 4,5,6: cord blood; Lane 7,8,9: Liver ALP; Lane 10,11: placental ALP; Lane 12,13,14: U2OS extract; Lane 2,5,8,13 sample preheated at 56°C for 10 min; Lane 3,6,9,11,14 sample preheated at 65°C for 6 min

ALP bands were visulized with its own enzyme activity by adding α-naphthyl-phosphate couple with diazonium salt, products are insoluble reddish brown precipiate.

The pH gradient of the gel was determined by the protein pI markers run parallel with the samples

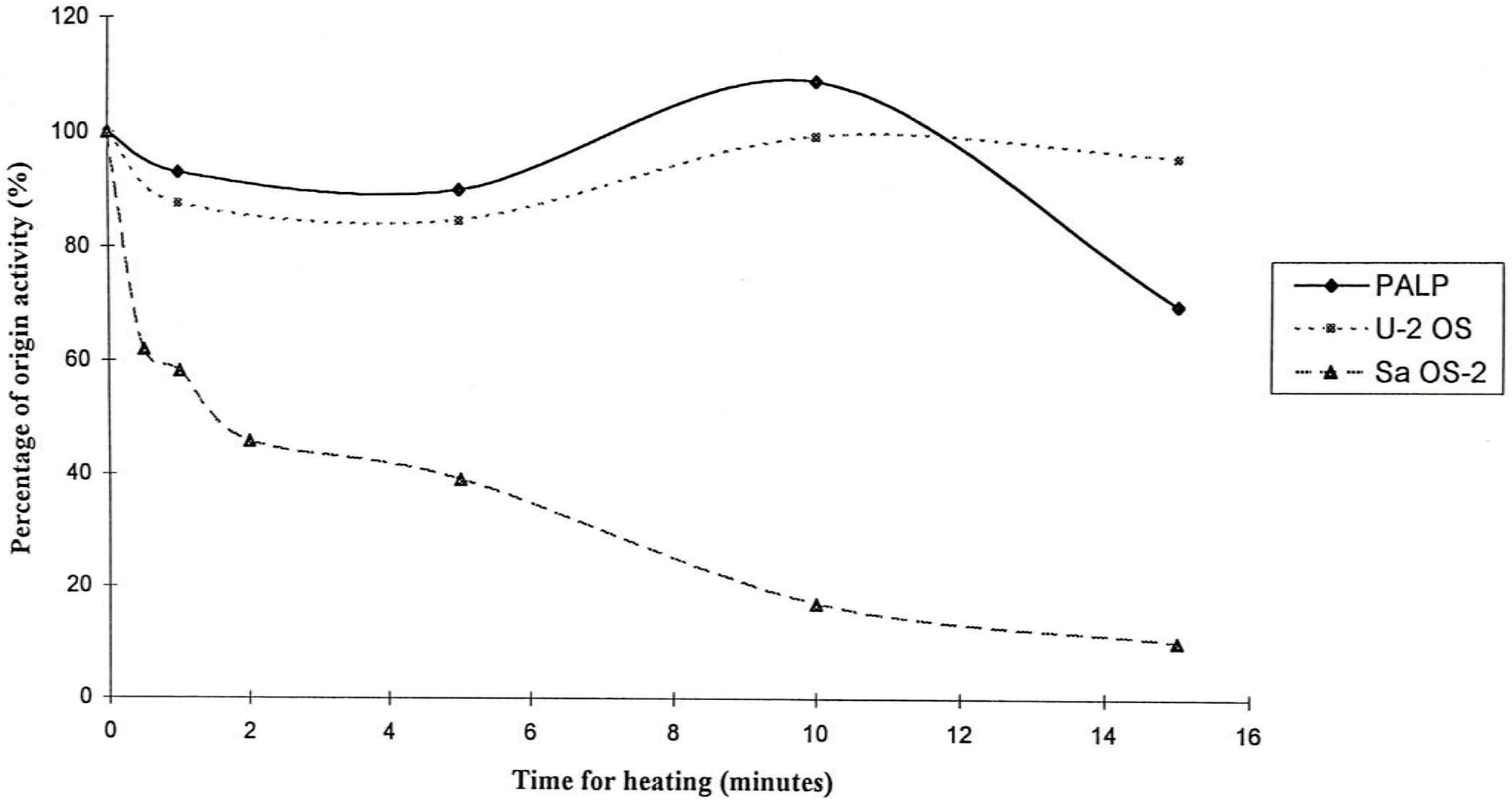


Figure 3.24. 56C heat inhibition study on ALPextracted in human placenta, U-2 OS & Sa OS-2 Cell

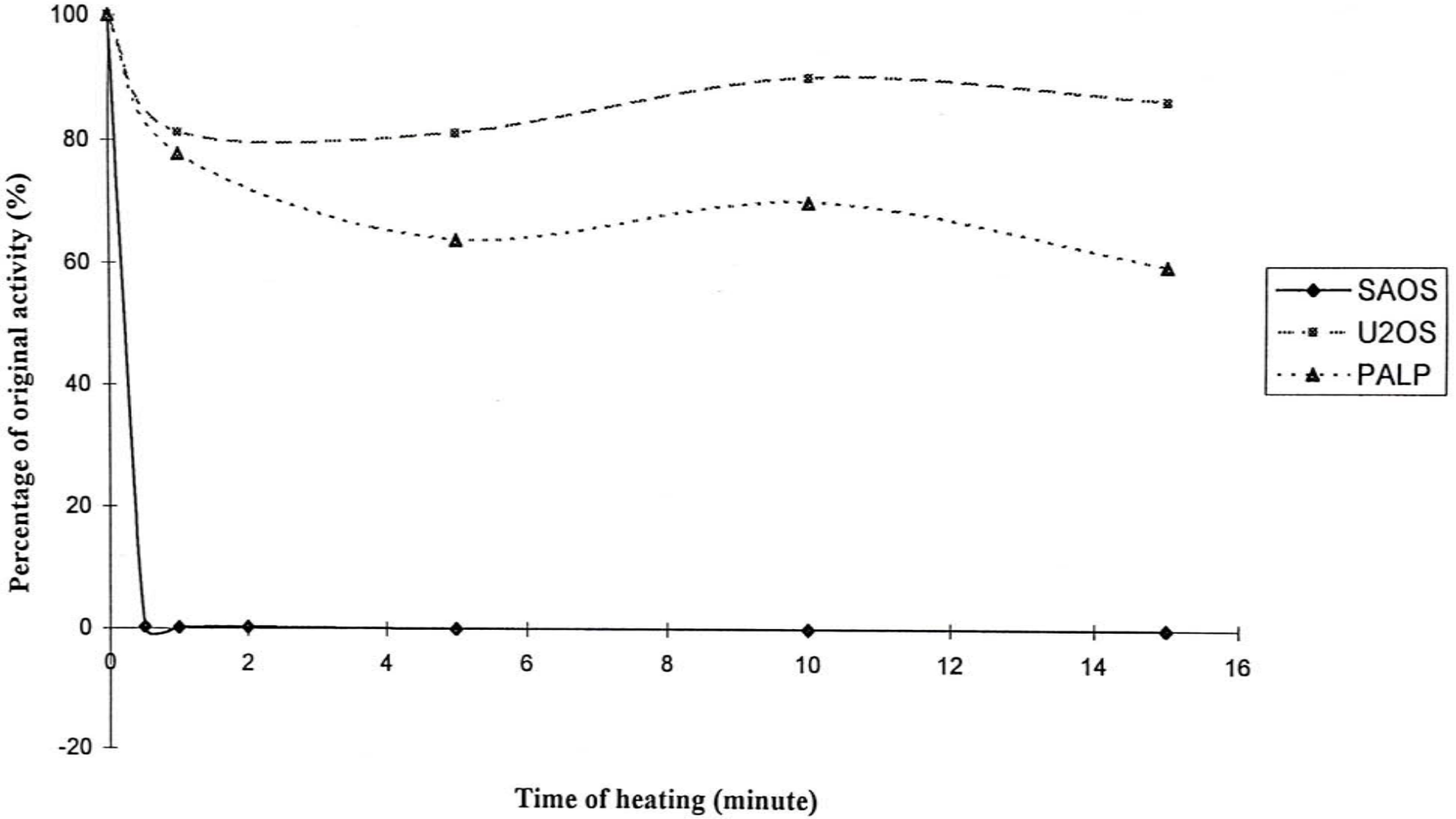


Figure 3.25. 65C heat inhibition study on ALPextracted in human placenta, U-2 OS & Sa OS-2 Cell

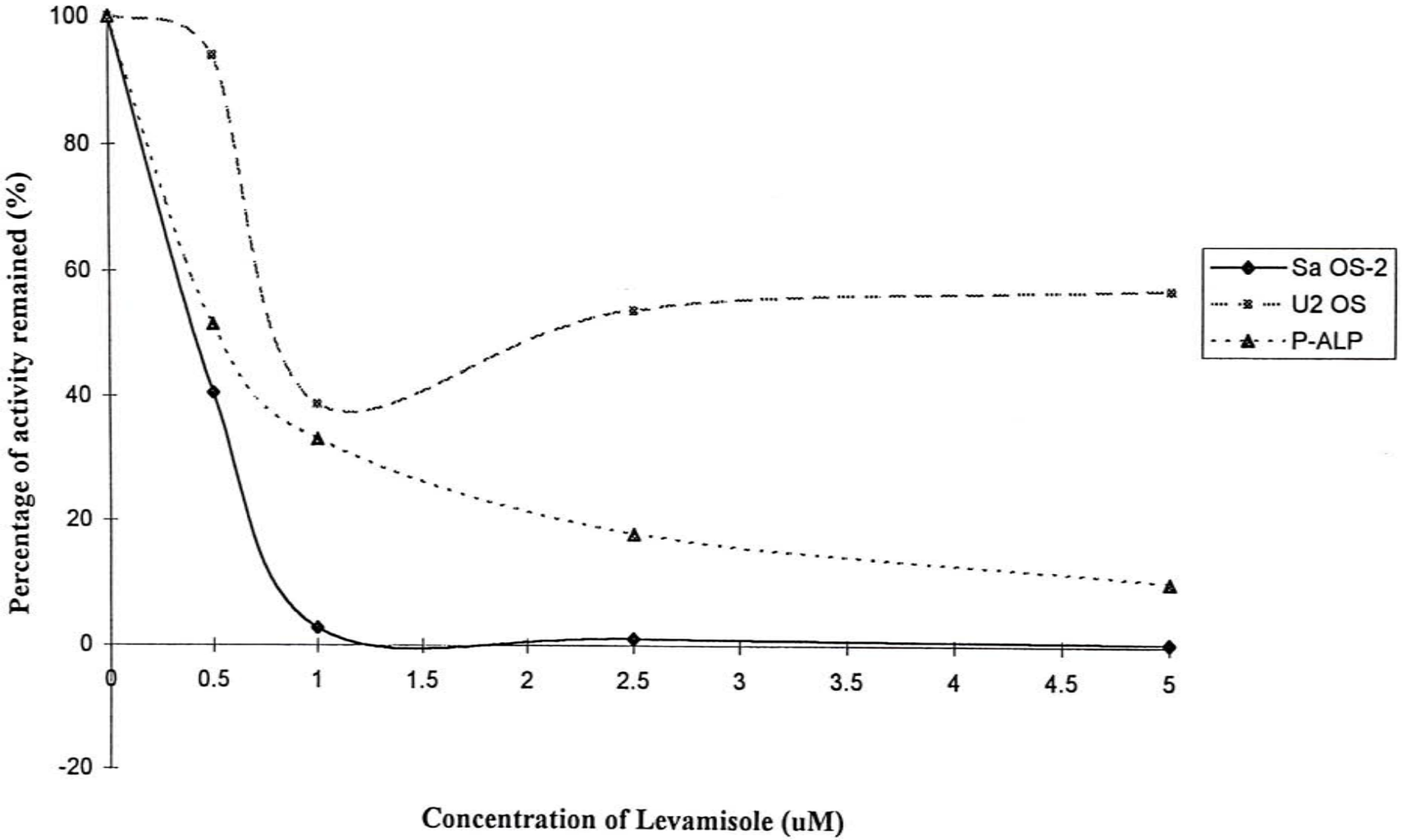


Figure 3.26. Levamisole inhibition study on ALP extracted in human placenta, U-2 OS & Sa OS-2 Cell

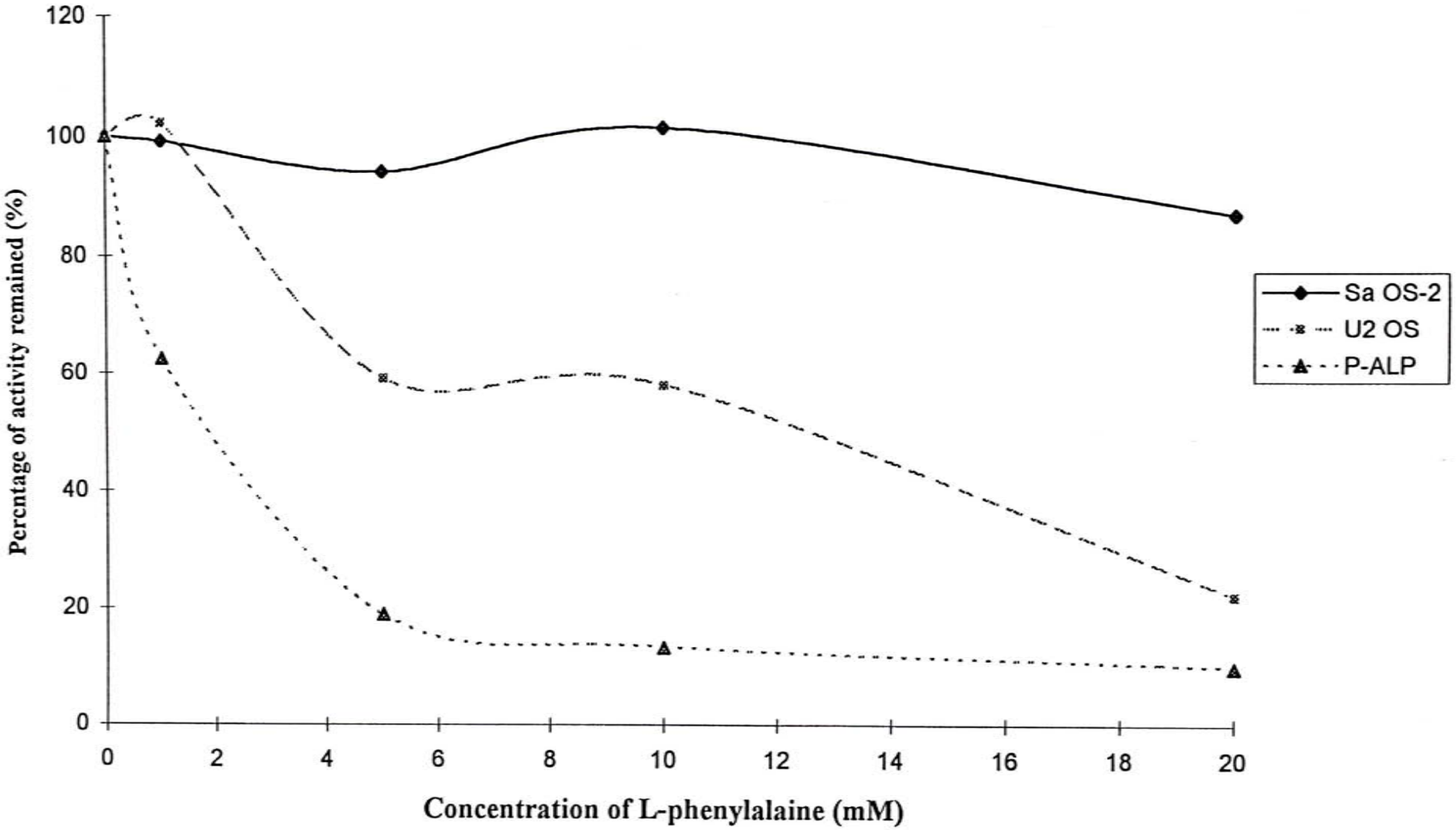


Figure 3.27. L-phenylalaine inhibition study on ALP extracted in human placenta, U-2 OS & Sa OS-2 Cell

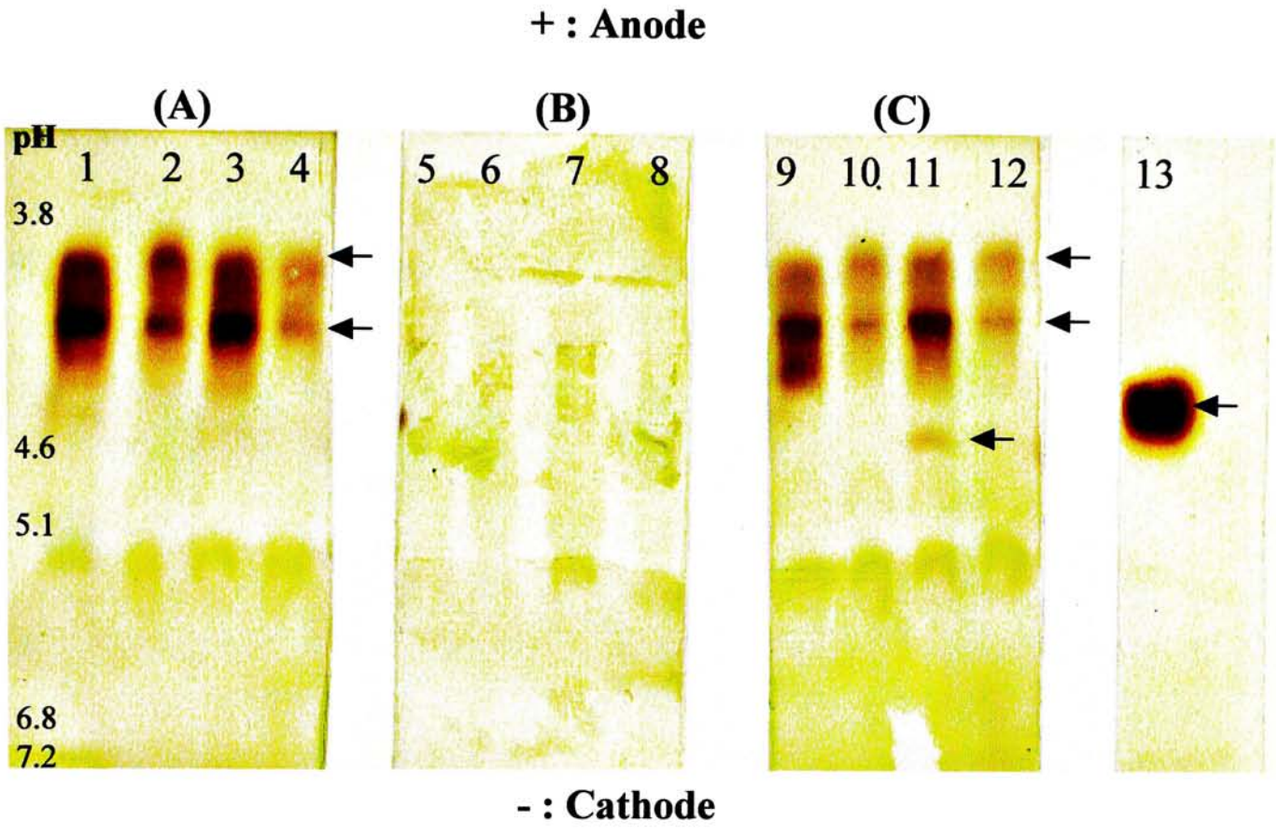


Figure 3.28. IEF separation of osteosarcoma patient plasma samples (A) No pre-treatment; (B) Samples pre-heating at 65°C; (C) Staining solution containing 0.06 μM Levamisole.

Lane 1,5,9: Plasma sample from YCY; Lane 2,6,10: Plasma sample from LYM; Lane 3,7,11: Plasma sample from BWY; Lane 4,8,12: Plasma sample from HHW, Lane 13: placental ALP standard

All samples added were diluted to around 100U/L before the separation

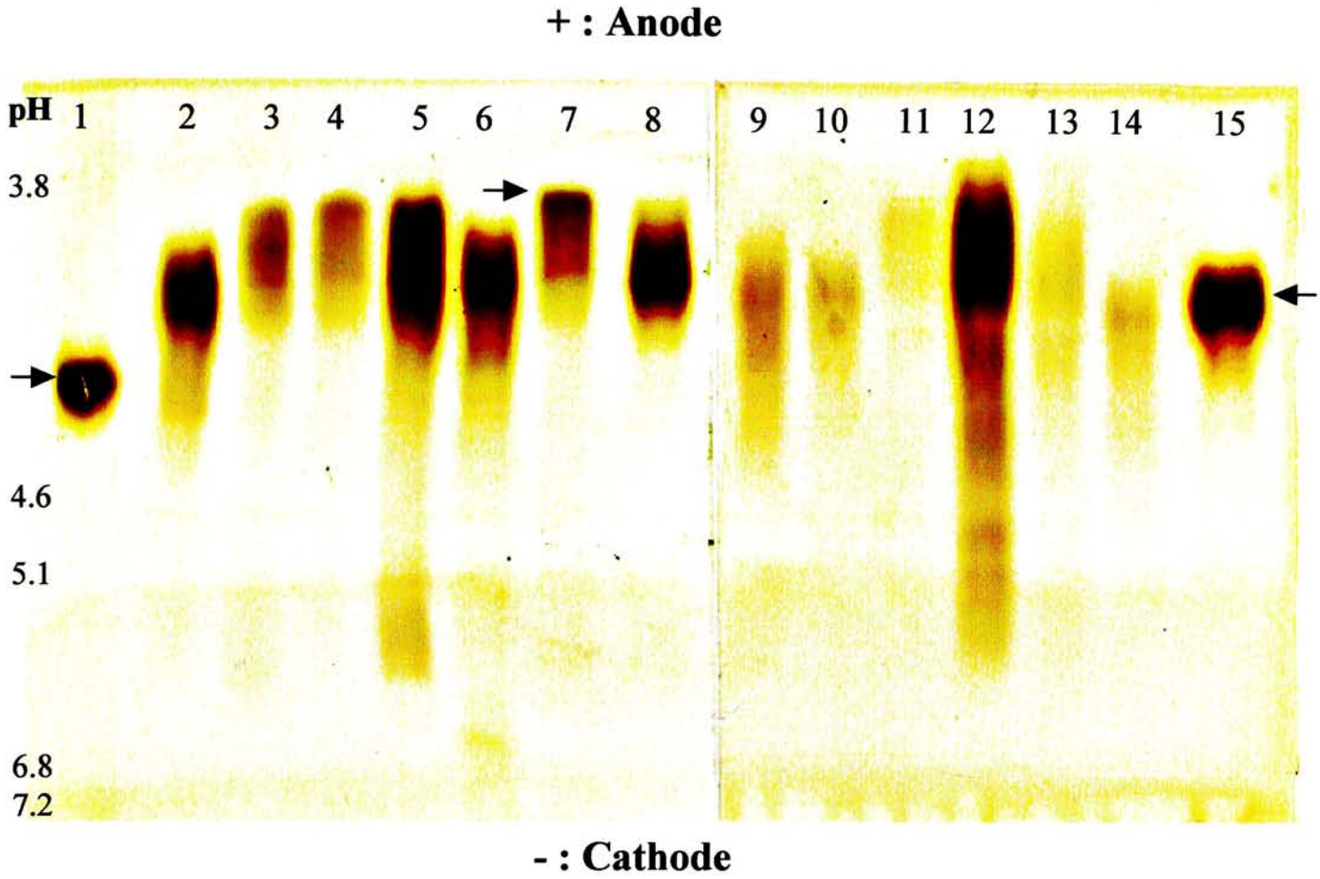
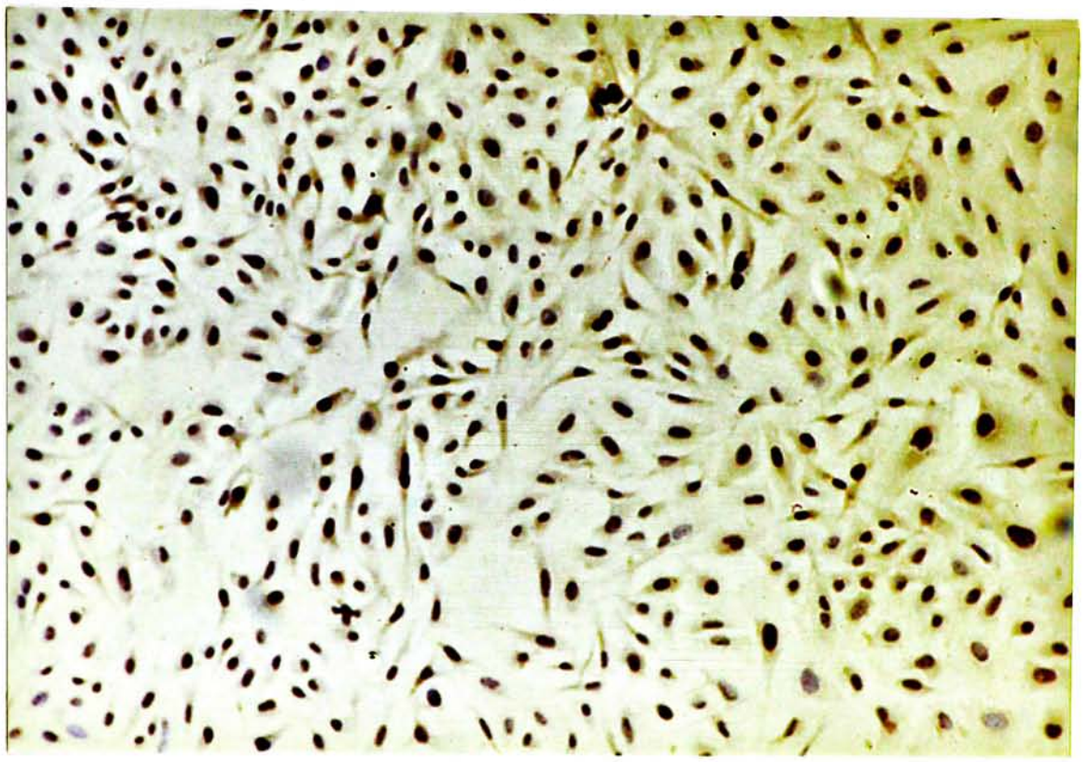


Figure 3.29. IEF separation of osteosarcoma tissue ALP extracts
Lane 1: Placental ALP standard; Lane 2: BWY ALP extract; Lane 3: KSW ALP extracts; Lane 4: MCW ALP extract; Lane 5: SHL ALP extracts; Lane 6: MLK ALP extract; Lane 7: Liver ALP standard; Lane 8: CMS ALP extract; Lane 9: LDK ALP extract; Lane 10: YCY ALP extract; Lane 11: WCW ALP extract; Lane 12: CKL ALP extract; Lane 13: HSF ALP extract; Lane 14: HHW ALP extract; Lane 15: BALP standard.
All samples with initial activity > 100U/L before the separation.

(A)



(B)

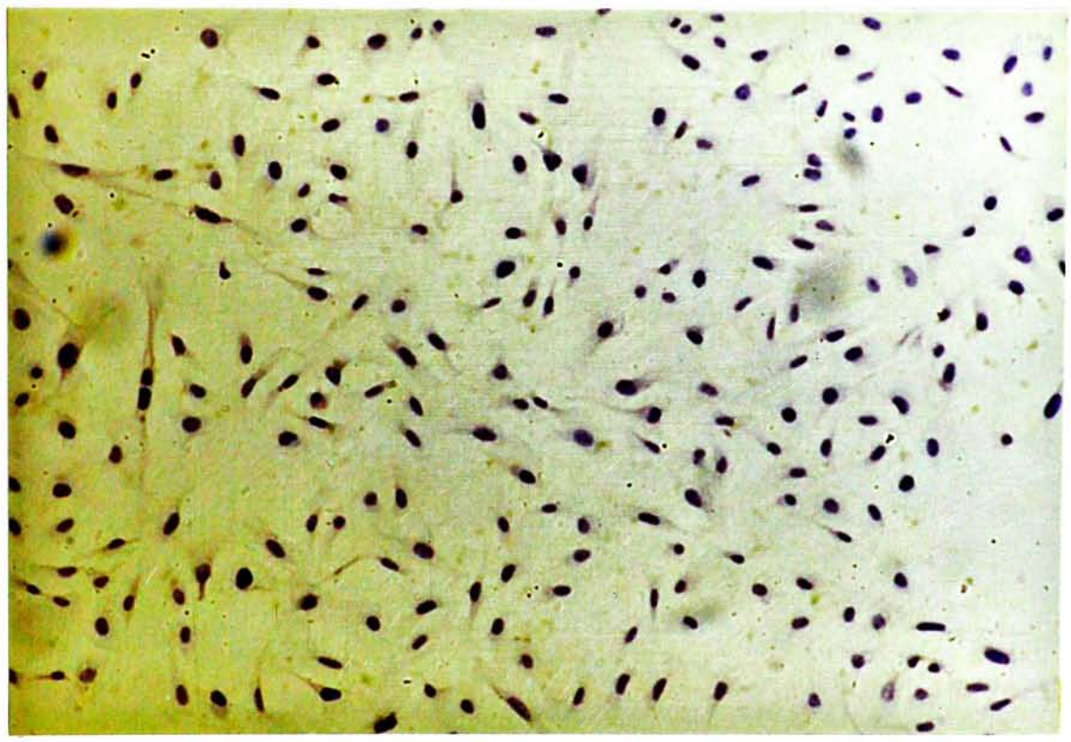
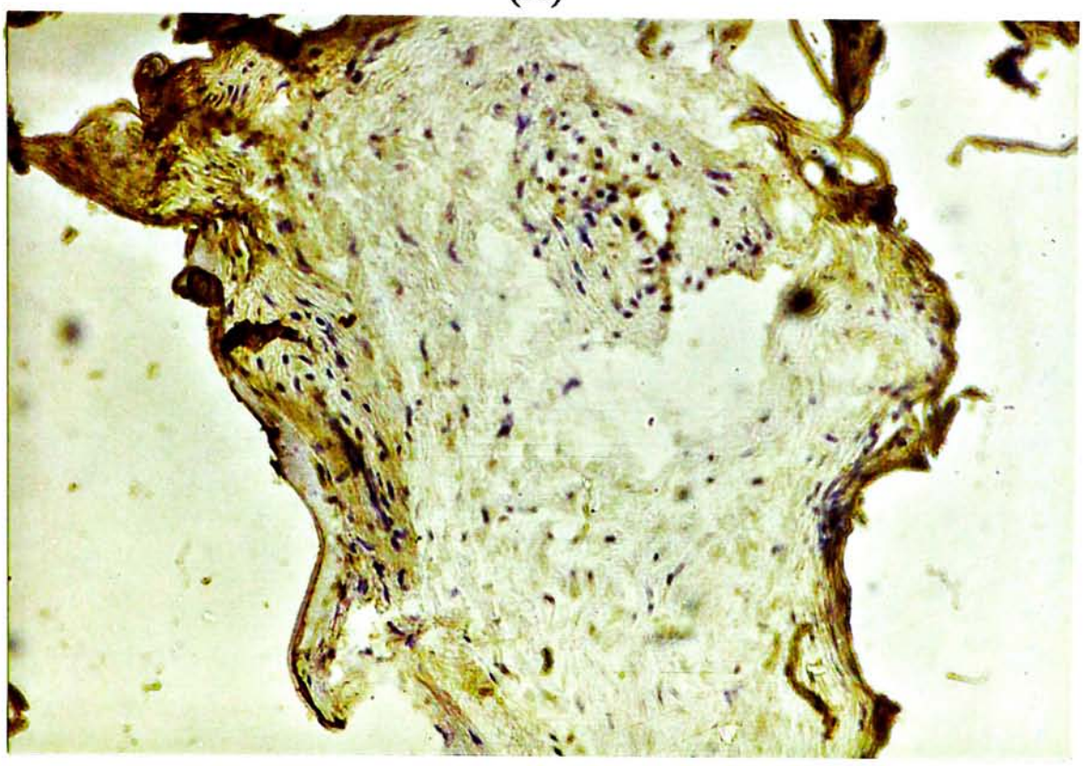


Figure 3.30. Photographs showing the immunohistostaining of placental ALP on seeded U-2 OS cells, using monoclonal antibody against human placental ALP as primary antibody (×200, counterstain by hematoxylene).

(A) With overnight incubation of primary antibody.

(B) Primary antibody incubation was replaced by 1%BSA/PBS solution.

(A)



(B)

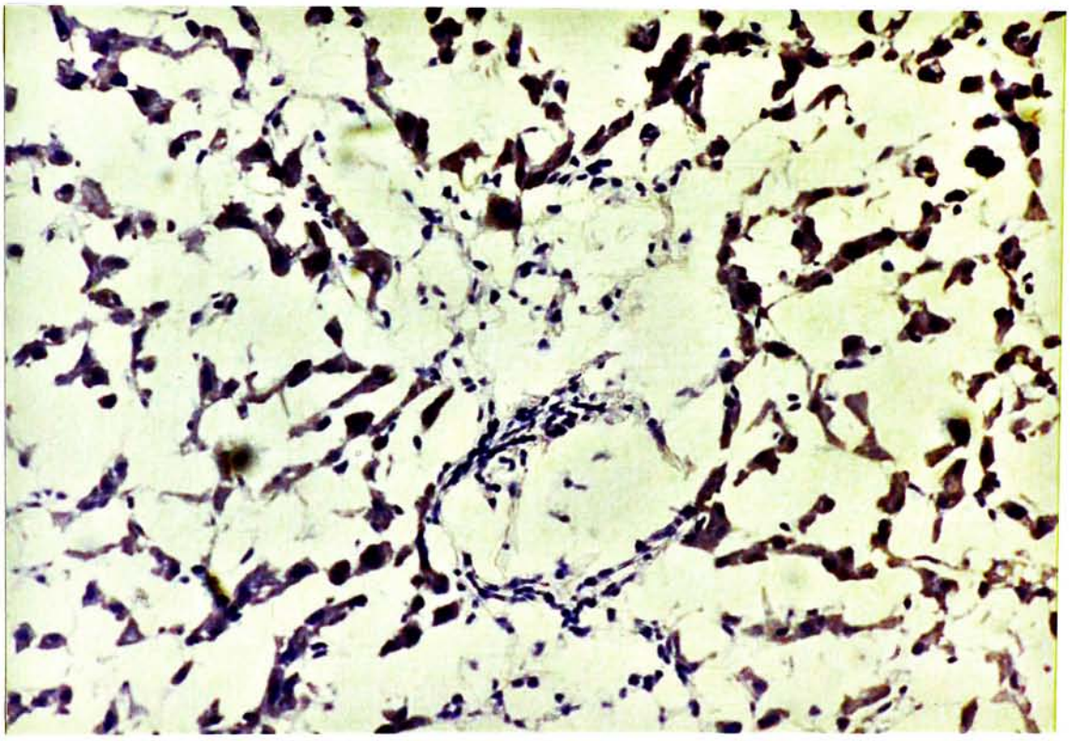
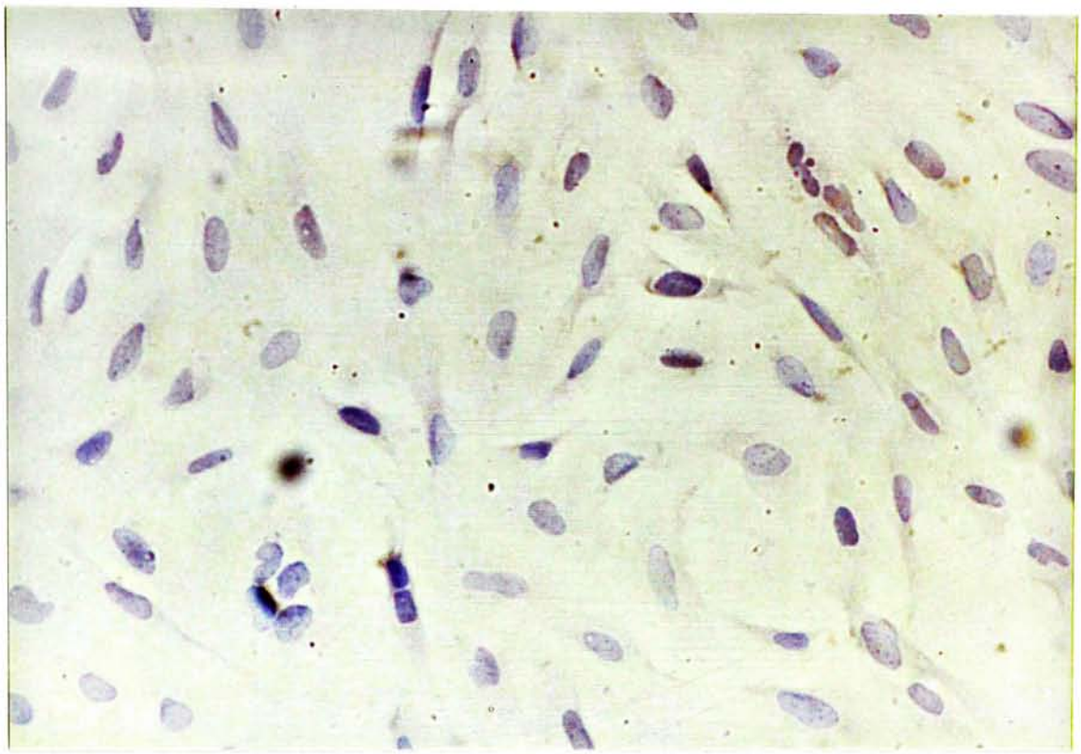


Figure 3.31. Photographs showing the immunohistostaining of placental ALP, using monoclonal antibody against human placental ALP as primary antibody on :
(A) Human placenta tissue section (×200)
(B) Human liver tissue section (×200)
All sections counterstain with hematoxylin

(A)



(B)

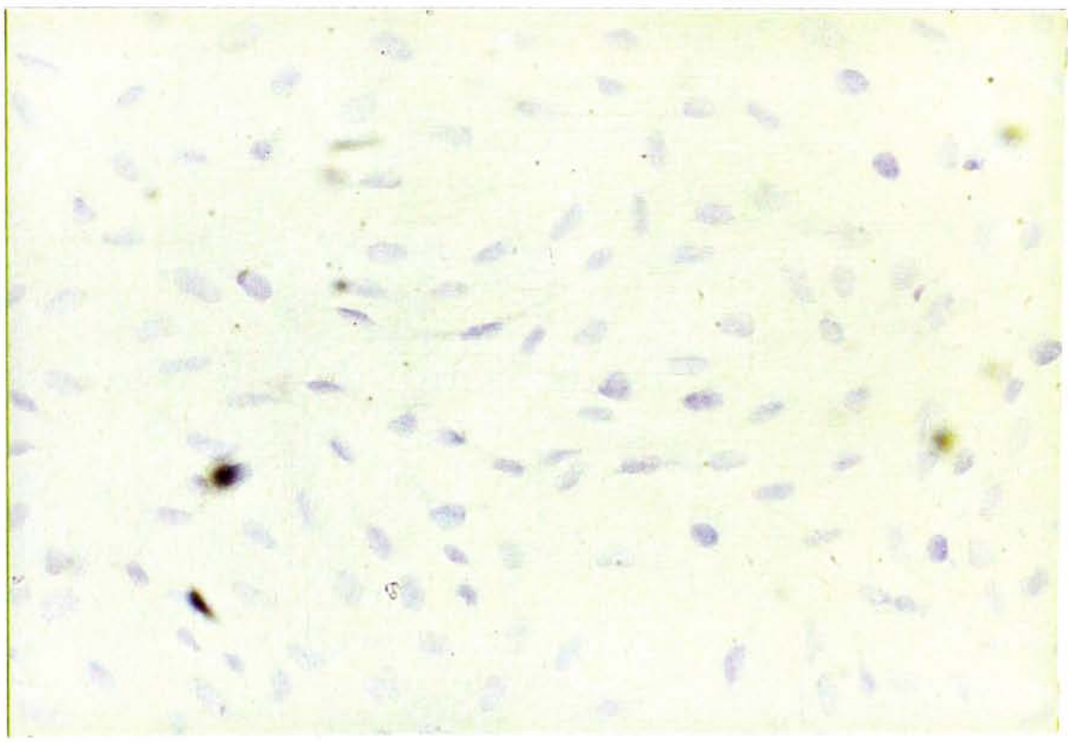


Figure 3.32. Photographs showing the immunohistostaining of placental ALP on seeded Sa-OS-2 cells, using monoclonal antibody against human placental ALP as primary antibody (×200, counterstain by hematoxylene).

(A) With overnight incubation of primary antibody.

(B) Primary antibody incubation was replaced by 1%BSA/PBS solution.

(A)

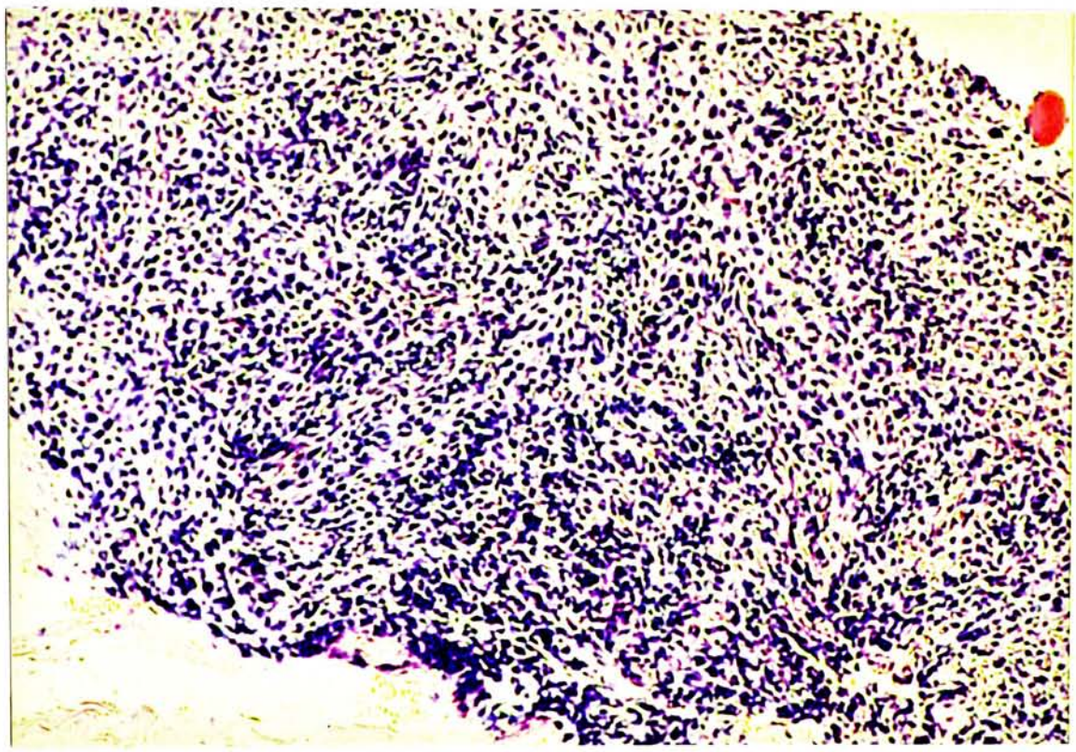


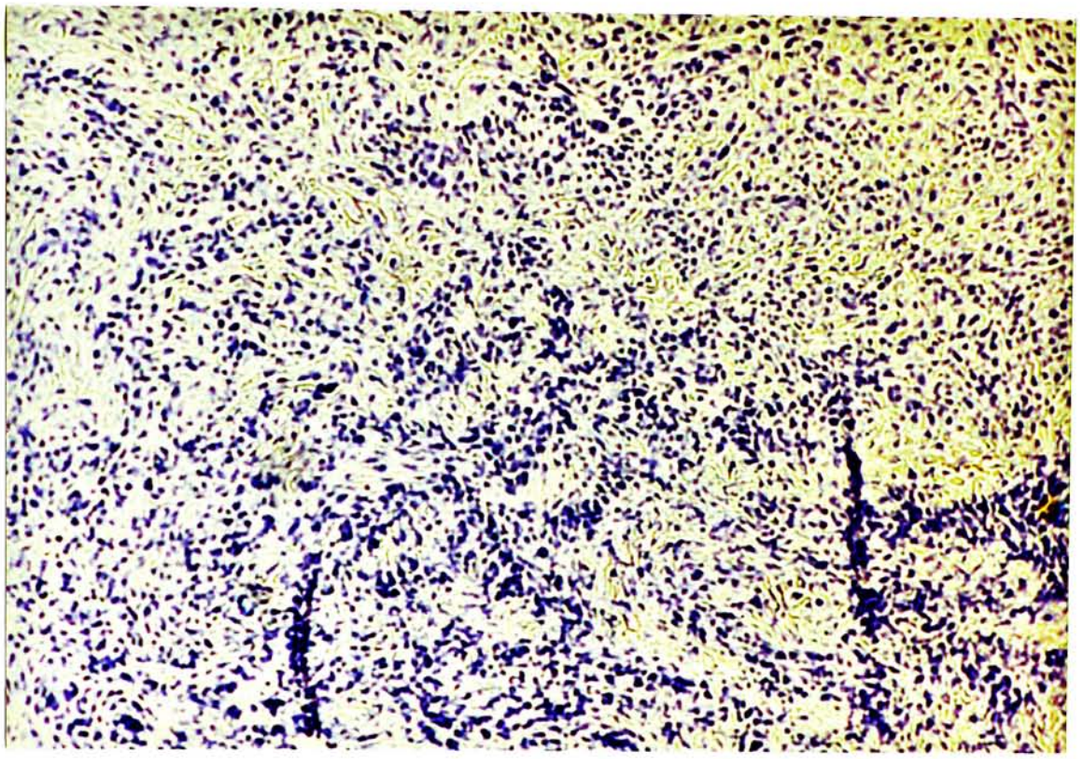
Figure 3.33. Photographs showing the H&E and immuno-histostaining of placental ALP (counterstain with hematoxylin) on patient CSL osteosarcoma tissue sections. (×100)

(A) H&E staining

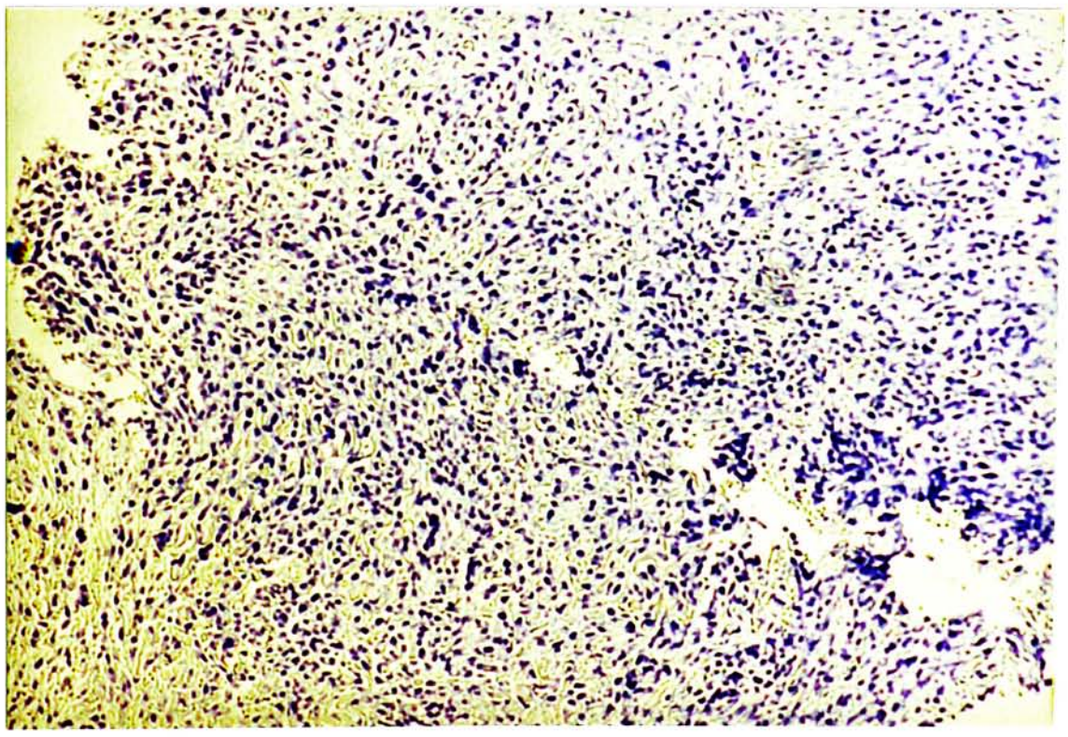
(B) With overnight incubation of primary antibody

(C) Primary antibody incubation was replaced by 1%BSA/PBS solution

(B)



(C)



(A)

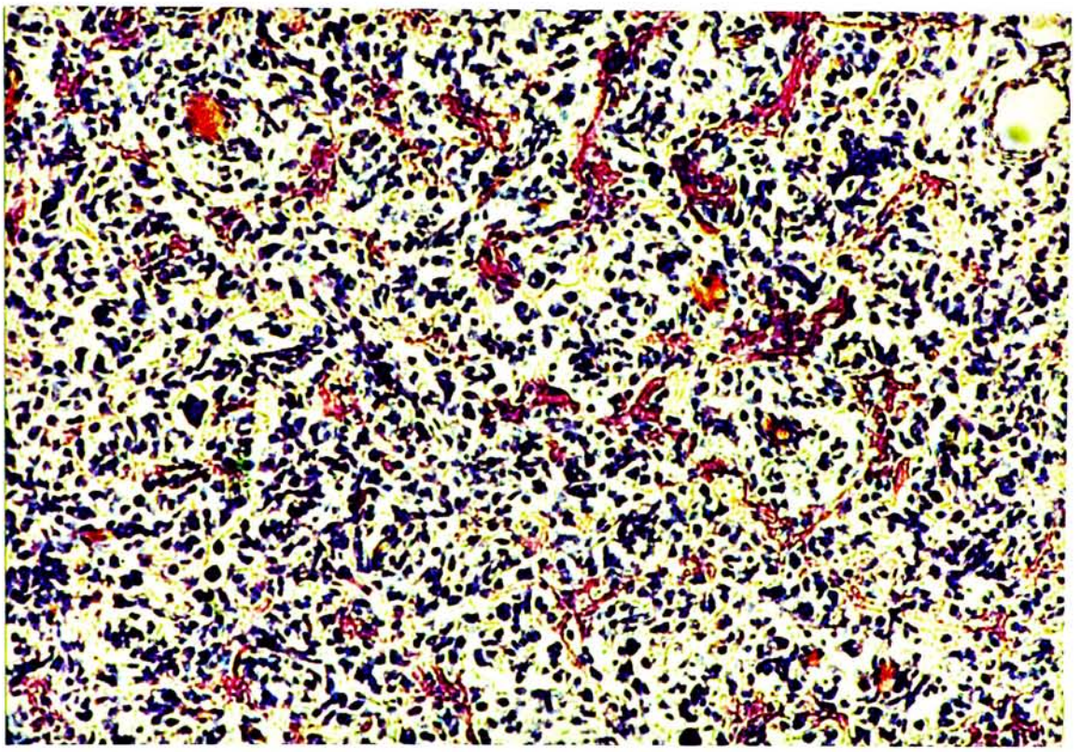


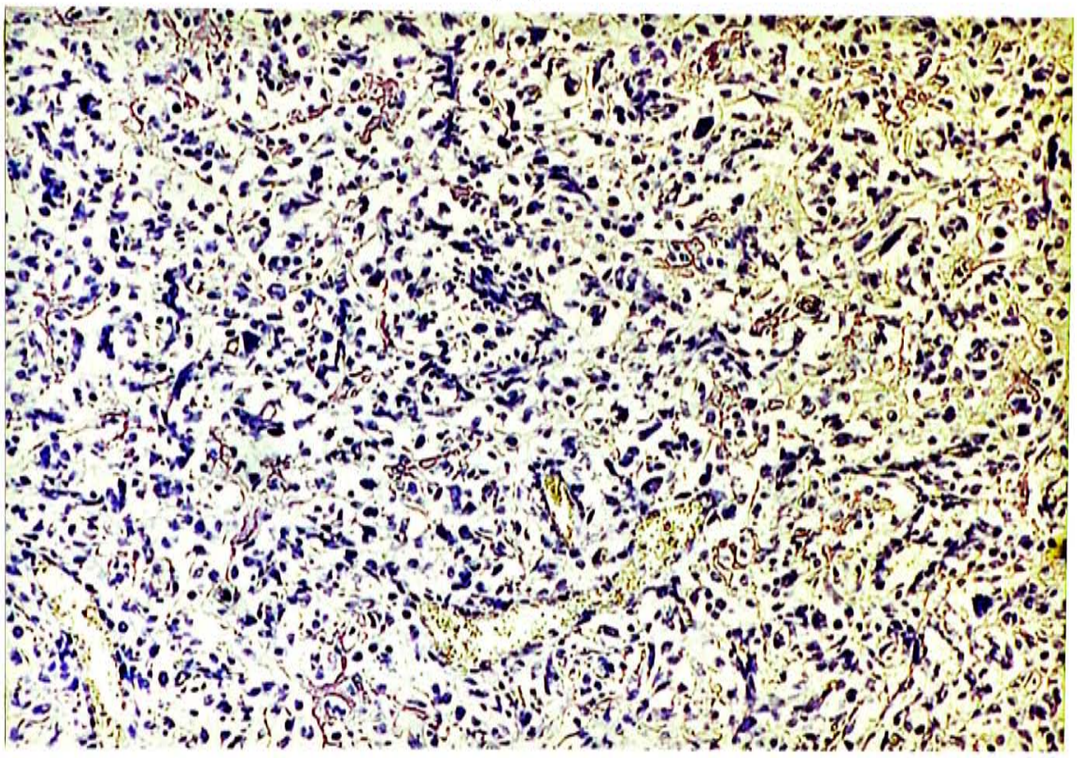
Figure 3.34. Photographs showing the H&E and immuno-histostaining of placental ALP (counterstain with hematoxylin) on patient WCW osteosarcoma tissue sections. (×100)

(A) H&E staining

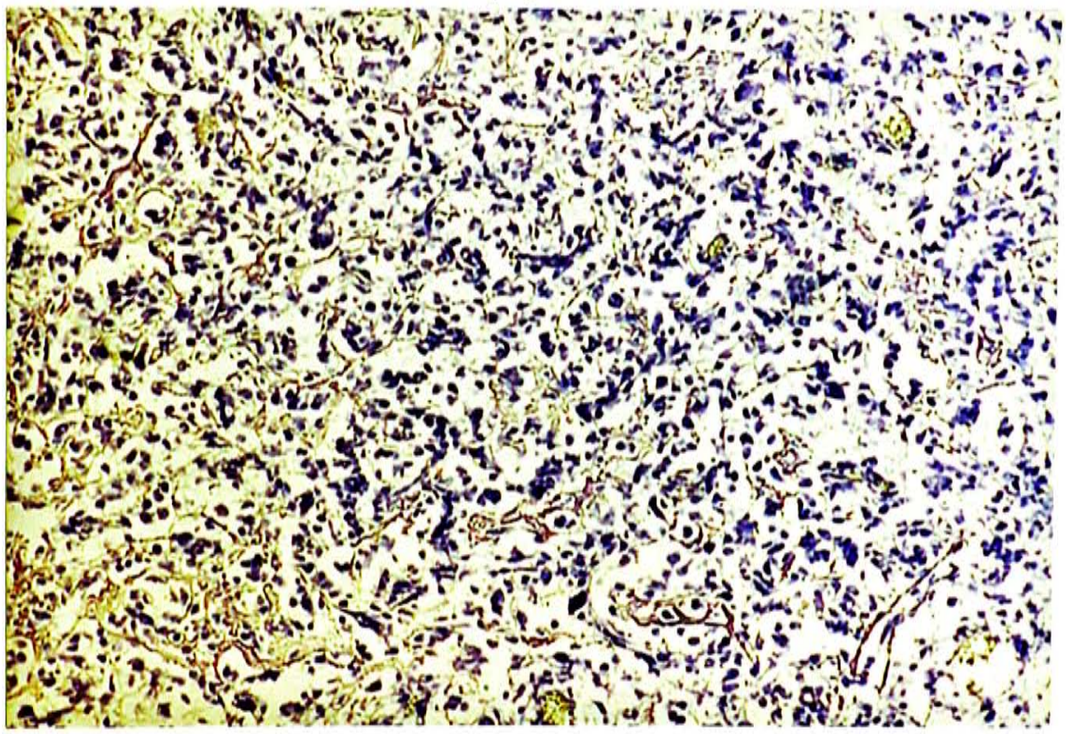
(B) With overnight incubation of primary antibody

(C) Primary antibody incubation was replaced by 1%BSA/PBS solution

(B)



(C)



Chapter Four: Discussion

4. Discussion

In the 1960s, osteosarcoma was considered as an incurable disease. Even with complete local tumor control by amputation, the survival rate of osteosarcoma was still a disappointing 20%. Multiple metastasis was the most commonly encountered problem and it nearly always led to an extremely poor outcome and early death.

It was not until the introduction of multiple drug regime in 1970s, i.e. the use of several chemotherapy drugs spontaneously for cancer treatment, that the disease free survival rate were improved dramatically. The use of both neoadjuvant and adjuvant chemotherapy successfully eliminates mircometastasis to the lung and to other areas. In addition, chemotherapy can create a more clearly surgical margin around the tumor, making limb-sparing surgeries possible. With all these recent advances in treatment of osteosarcoma, both the clinical and functional outcomes of osteosarcoma patient are greatly improved.

With increasing long-term survival rates in osteosarcoma patients, monitoring of the disease progression now becomes very important and raises the need for a sensitive biochemical marker of the disease. Such a sensitive biochemical marker not only can allow close monitoring of the treatment progress but also helps diagnosis and provides several prognostic insights into the disease.

Up to date, the most commonly used biochemical marker in osteosarcoma is the plasma total ALP. As a biochemical marker, ALP has the advantage of easy and well define methods of measurement and is readily detectable in either serum or plasma samples. Many investigators demonstrated that plasma total ALP activity has prognostic values (Bacci *et. al.*, 1995) and can closely reflect the treatment progress of the disease (Davie *et. al.*, 1991).

Despite of these encouraging findings, plasma total ALP measurement has its own limitation: its level is greatly affected by other factors. Circulating plasma ALP is mainly contributed by two sources: the liver origin and the bone

origin. Thus, measured plasma total ALP level is the reflection of these two ALP isoforms. In osteosarcoma patients, bone ALP isoform is usually elevated and therefore allowing the total ALP level to be used as a biochemical marker. However, in patients with liver disease or having liver toxicity during the chemotherapy, the plasma total ALP level will also be elevated by the liver ALP isoform. This elevation will lead to a misleading estimation of the disease.

Measurement of the plasma BALP alone will avoid this misleading result. In the past, the use of plasma BALP measurement is primary limited by the difficulty in differentiate it from the liver ALP isoform. Only semi-quantitative measurement of plasma BALP is possible by heat inactivation at 56°C and 65°C. An improved method of plasma BALP measurement is now possible by WGL precipitation method (Rosalki & Foo, 1984) and also the newly developed ELISA kit using monoclonal antibodies against the human bone-specific ALP. The availability of these improved methods of plasma BALP measurement give scientists chance to explore the clinical usefulness of this newly developed marker in osteosarcoma.

A total of 49 osteosarcoma patients were recruited for our study. The age and sex distribution of the patients is shown in Figure 3.1. In our series, the tumor occurred more often in males, with more than half (65.3%), which agrees with the general male predominance in most other studies (Dahlin, 1986; Campanacci, 1990; Huvos, 1991). In addition, affliction of the young is also observed in which most cases occurred in the second decade of life (61.2% of total occurrence). Concerning the site of osteosarcoma, most of the affected sites are around the actively growing physeal plate (knee joint area, i.e. the distal femur and the proximal tibia).

With respect to the above descriptions, the general information of the osteosarcoma patients in the local Chinese population matches the descriptions with the results obtained in most of the studies conducted in other countries.

Measurement of plasma BALP was done by several methods in this study. In order to compare the data measured by different methods, the correlation of different measurements were done and shown in Figure 3.2 and Figure 3.3. All data were converted into unit, which is compatible to ALKPHASE-B® ELISA kit. High correlation was found in the ABBOTT methods Vs. ALKPHASE-B method and the COBAS MIRA method with the ALKPHASE-B® methods, with both R^2 above 0.9.

Since the use of plasma BALP measurement is not popular in Hong Kong, there is no normal reference range of plasma BALP in local Chinese population. Leung, *et al* in 1993 established the normal reference for the local Chinese population. In that study, they showed that the plasma BALP activity in normal Chinese children is high in children, declines rapidly in adolescence and remains low in adults. The mean value of the plasma BALP activity for adult males is 39.76 ± 16.68 U/L and for adult females is 31.36 ± 12.41 U/L. The range is 23 to 56 U/L. However, in that study, only the normal reference for adults was established. Since most of the osteosarcoma patients are children or teenagers, comparison of their plasma BALP level with the adult normal reference will be inappropriate. Nevertheless, the plasma BALP activity in children and in adolescence has to be reestablished.

The normal reference of the local Chinese population in differenct age groups is listed in Table 3.4. Normal is defined as "no past history or present sign or symptoms of liver or metabolic bone diseases". Normal subjects were divided into 3 groups according to their ages. The three groups are N1 with age below 12, N2 with age between 12 to 16 and N3 with age above 16. The definition of above age groups depends on the normal growth of man, with 2nd sexual development at about age 12 and reach maturity at about age 16. There is significant difference in normal reference of the three age groups, with highest values in childhood then adolescence and lowest in adults (Figure 3.4). Since BALP is an important enzyme responsible in bone formation, the elevation of plasma BALP in children and adolescence reflects their active

growth periods in the skeletal structure. In addition, since the normal plasma BALP level in different age group are significantly different from each other, it is important for us to compare the plasma BALP level in osteosarcoma patients with an age-matched normal reference.

Plasma BALP level at admission was used to represent the initial BALP level of the patient before any treatment. When we compared the plasma BALP-adm with the normal subject independent of age group, we found that plasma BALP-adm is significantly higher than the normal plasma BALP. Similar results were obtained when using plasma total ALP-adm level. When plasma BALP-adm of patients were compared with age-matched normal groups, significant differences were only found in T2 ($p < 0.01$) and T3 ($p < 0.001$) groups (figure 3.7 & 3.8). Thus, plasma BALP-adm can be used as a diagnostic tool for patients with age above 12.

For age below 12, there is no significant difference between the plasma BALP level in normal subjects and the osteosarcoma patients (Figure 3.6). It may be due to the fact that the plasma BALP level in this age group is already very high, the increase of the plasma BALP in osteosarcoma patients is not so significant with the initial high baseline value. Thus, plasma BALP-adm may not be a good diagnostic tumor marker as in other age groups.

Compared with the normal subjects, plasma TALP-adm level of the patients are also significantly higher than the normal, independent of age. However when age-matched comparison was performed, significant difference in plasma TALP can only be demonstrated in T3 group (Figure 3.12). In other 2 age groups, plasma TALP level fails to show a statistically significant difference between the normal and the patients. Thus, in term of a diagnostic tool for osteosarcoma, plasma BALP level is more sensitive than using just the total plasma ALP level.

To improve the disease-free survival, it would be important to identify patients with poor prognosis who could be treated with more aggressive therapy. Pretreatment plasma total ALP level is considered as one of the

prognostic factors of osteosarcoma (Bacci *et. al.*, 1993). Patient with increased pretreatment plasma ALP level is significantly higher in the metastatic group than in the group of patients with localized disease. Moreover, among patients with elevated plasma TALP levels, the percentage of relapses was higher in patients with high levels of the enzyme in comparison with patients with moderately elevated values.

The possibility of using plasma BALP-adm for prognosis assessment is investigated. Plasma BALP-adm successfully reflects patients with higher chance of local recurrence and longer survival periods. Patients with local recurrence of osteosarcoma after operation removal of the lesion have significantly higher plasma BALP-adm value compared with the non-recurrence patients (Figure 3.13).

In addition, the plasma BALP-adm levels were compared with respect to the survival rate of the patients. The plasma BALP-adm of patients whose passed away within 3 years, had significantly higher plasma ALP-adm level compared with the disease-free patients. Moreover, the two tail significance difference of plasma BALP-adm gradually decreased with increasing survival years, indicated that the higher the plasma BALP-adm, the poorer the prognosis of the disease is.

The plasma BALP-adm can directly reflect the tumor burden of the patients. Plasma BALP-adm shows a positive correlation with the tumor volume (Figure 3.17), i.e. with higher plasma BALP activity, the volume of the tumor is higher. Since the BALP is released directly from the tumor, it is reasonable to believe that with an increase in tumor volume, the amount of BALP produced by the tumor cell increased. However, though the correlation is significant, the correlation coefficient is only 0.6127. This, rather, poor correlation between the plasma BALP level and the tumor volume may be affected by the subtype of the tumor. Even with about half of the osteosarcoma cases are classified as "osteoblastic" (Dahlin, 1978), other subtype of osteosarcoma may not have large amount of bone production, sometime may

even be osteolytic. Moreover, the measurement of tumor volume include the whole lesion area, i.e. some part of measurement are the reactive zone of the tumor but not the tumor cell itself, leading to an over-estimation of the actual tumor volume.

As an effective biochemical marker for tumor, perhaps the most important criteria are the use of the marker for the monitoring of the therapy and to answer the commonly asked questions: Can the marker tell the physician whether therapy has been effective? Does normalization of the marker indicate that all malignant disease has been eliminated? For plasma BALP, we demonstrated that it reflects the successfulness of the pre-operative chemotherapy.

With a successful chemotherapy, most of the tumor cells should be kill by the chemotherapy drugs. Based on the histological studies of the resected specimen, the pathologist determines the percentage of necrosis of the tumor killed by the drugs. For patients with drops of plasma BALP level back within the age-matched normal range during the chemotherapy ("DROP" group), their degree of tumor necrosis is significantly higher than patients without the drops of plasma BALP ("REMAIN" group).

The use of plasma BALP to estimate the successfulness of the chemotherapy is important. It can provide a useful information to the physician or the surgeon about to tumor itself so to plan for a better regime for the patients.

For patients with good responses toward the pre-operative chemotherapy (above 80% tumor necrosis), their elevated plasma BALP level significantly drops during the first few weeks of chemotherapy. In addition, the plasma BALP level always drops after the removal of the tumor (Figure 3.19). This drop of plasma BALP is not always observed in those patients with poor response to the chemotherapy (degree of tumor necrosis below 50%)(Figure 3.20). In fact, most of poor response patients show no significant drops of plasma BALP, their plasma BALP, especially for patients with very high level

of enzyme activity, show great fluctuations during the chemotherapy. In these patients, the clinical outcome is usually much poorer than the others. Patients KSW, SC and TI all have initial high and fluctuated plasma BALP activity. SC and TI passed away within half year of the operation and KSW died even before the operation removal of the tumor. Moreover, the plasma BALP level did not drop back to the normal range even after the surgical removal of the tumor. Since most of the BALP was produced by the tumor cells, the elevated plasma BALP after the operation may imply incomplete removal of the tumor or the presence of undetected metastases. In our 2 cases, the former reason should not be the case since pathological examination show clear margin of the resected specimen. Thus, the high plasma BALP level was most probably caused by the already metastases tumor cell which did not response to chemotherapy and have not been resected during the surgery.

Plasma BALP level can be used to guide the response of the patients towards the chemotherapy. Patients with drops of the plasma BALP level usually have a better response toward the chemotherapy. On the other hand, patients which have a large fluctuation in plasma BALP level usually represent a more poor response toward the chemotherapy and extra care should be paid to those patients. Change of present chemotherapy regime should be considered. Plasma BALP level, after the surgical removal of the tumor, also play an important role in identify patients with high risk. If the patients' plasma BALP level did not drop back to normal reference even after the removal of the tumor, careful examination of the surgical margin to check the successfulness of the operation or any metastasis should be done. As demonstrated in TI case, his plasma BALP level did not drop back to normal range after first operation on the extremity but only after the surgical removal of the lung metastases. More efforts should be paid on the detection and removal of the secondary metastases so to avoid lethal local recurrence of distant metastasis.

Another important role of plasma BALP level is to detect early local relapse of the disease. Figure 3.21 shows that plasma BALP level raise during the detection of recurrence. Most of the patients with their plasma BALP level elevated above the normal reference during the time of relapse. For patients TI and YCY, the elevation of plasma BALP level were detected 2 months before the clinical confirmation of the local recurrence. However, not all elevation of the plasma BALP level during the follow-up periods necessarily implied local relapse. Bone reaction or fracture in the bone allograft may also lead to an raise in plasma BALP level. Thus, when encounter elevation in plasma BALP level, careful examination should be done to rule out other possibilities.

In summary, plasma BALP is a good biochemical marker in osteosarcoma. The pretreatment plasma BALP level can provide diagnostic aid and also prognostic insight to the physician. In addition, the change of plasma BALP level during the pre-operative chemotherapy can reflect the successfulness of the treatment and therefore provide important clue to the physician how the patients is responding and whether it is necessary to change the plan of treatment. Plasma BALP level is also a valuable tool in follow-up period as it can detect early metastasis and thus may allow an early treatment to the relapse.

Placental ALP is synthesized in the syncytiotrophoblast cells of the placenta from eight week of gestation throughout the pregnancy, which is distinct from the isozymes of other organs, and is found in the serum of pregnant women. In 1968, Fishman described the Regan isozymes, detected in the serum and tumor tissue of a patient with lung carcinoma that appears to be identical to the placental isozyme. Later, other isozymes of ALP were found to be tumor related and expressed in other carcinoma. However, no ectopic expression of any form of ALP is reported in osteosarcoma.

In 1993, Stinson *et al.* investigated the ALP mRNA expression in six human ostoesarcoma cell lines. He showed that 5 of the cell lines expressed only tissue-specific ALP mRNA. But in one of the cell line, U-2 OS, contains

a mixture of tissue non-specific ALP mRNA and at least one other form, indicated that there may ectopic expression of ALP in osteosarcoma. Therefore, we attempted to search for ectopic expression of ALP isozymes in osteosarcoma and try to explore any clinical uses of this ectopic expression.

There is a wide range of techniques available for the characterization and quantification of multiple forms of ALPs. The techniques depend on the physiochemical properties, the nature of the catalytic site or the protein structure of the different isoforms and any post-translational modifications. These methods include gel electrophoresis, isoelectric focusing, affinity precipitation, chemical denaturation, heat inactivation, chemical inhibition, chromatography and immunoassay. In our studies, isoelectric focusing electrophosis was employed for the separation of the ALP in plasma and tissue extracts. Resolution of protein mixture is much higher in IEF than conventional gel electrophosis and can reveal the microheterogeneity of ALP isozymes (Rosendahl *et. al.*, 1987). After the separation, the biochemical inhibition and heat inactivation properties of different ALP isozyme were explored for the characterization of ALP isozyme expression in plasma and tissue extracts.

Isoelectric focusing (IEF) separates proteins according to their pI point. The pI gradients of the gel we are using are ranging from 2.5 to 9.5. However, the gradient of the gel is not uniformed. Since at extreme pH, i.e. pH 2.5 and 9.5, the gradient of the gel is not stable. In order to stabilize the pH gradient of the gel and expanding the desired range of the pH gradients, we mixed ampholyte with pH range from 3 to 5 with the preblending Pharmalyte® pH 2.5 to 9.5. The pH gradient of the agarose IEF gel is shown in Figure 3.22. The pH gradient of the gel in pH range from 3.5 to 6.5 is quite even and the pH gradient sharply deepen after 6.5 since the addition of pharmalyte at pH 3 to 5 have expanded the original pH gradient and pulling the alkaline pH gradients deeper. As most of the ALP isozymes have their pI values lies in the acidic

range (2.8 to 5.2), with this modification of the pH gradients, the separation of different ALP isozyme can be more clear.

After the separation, ALP were directly visualized by it own enzyme activity. α-naphthyl phosphate were added in AMP buffer as substrate for ALP in which the product form couple with the diazo dye and form reddish brown insoluble complex. Heat inactivation and biochemical inhibition properties of different ALP isozyme were also employed together with IEF for the better characterization of ALP band visualized. Heating the sample in 65°C will destroy all forms of ALP isozymes except the placental ALP and germ-cell ALP. Heating with 56°C will preferably denature the BALP isoform as it is extremely heat sensitive. Moreover in some of the IEF gel, levamisole was added to the staining solution as an inhibitor of both the bone and liver ALP, thus allowing the visualization of other low level expressed ALP isozyme.

In Figure 3.23, we demonstrate the successfulness of the separation of different ALP isozyme standard prepared and also the cell extracts from the 2 cell line, U-2 OS and SaOS-2. Since all extracts are prepared by releasing the ALP from the membrane by PIPLC, the microheterogenicity difference caused by the GPI anchor in ALP isozymes is minimized.

Two human osteosarcoma cell line, U-2 OS and SaOS-2, were employed for the studies of the ectopic expression of ALP in osteosarcoma. ALP expression in SaOS-2 was well established, with sole production of bone-specific ALP (Nakamura *et al.*, 1988; Stinson *et al.*, 1993). With large amount of BALP production in SaOS-2 cells, Farley *et al.* in 1989 proposed that we could use the ALP extracts of this cell line as a standard for quantitative measurement of skeletal ALP activity in serum. Therefore we use this cell line as a source of pure BALP standard for the expression of ALP in both the patients' plasma samples and the tissue extracts

In order to confirm the expression of BALP in SaOS-2 cells, cord blood was used as a source of BALP. Rosalki & Foo in 1993 demonstrated that more

than 95 % of the ALP found in the cord blood are of bone origin. In lane 4 of Figure 3.23, one major band was observed in the acidic regions of the IEF gel. The BALP moved towards the anode and have pI value of around 3.9. This result is consistent with previous reports (Angellis *et. al.*, 1976; Sinha *et. al.*, 1986; Rosendahl *et. al.*, 1987). Sa OS-2 cell extracts also show a similar band in the same position. Moreover, the ALP pattern in SaOS-2 cell extracts have the same heat inactivation properties with the cord blood, after a short heating at 56°C, all the ALP activity in both samples disappeared. Comparing with the liver ALP standard prepared, complete deactivation of ALP only occur after 65°C heating and the position of the band is located in a more acidic pH with pI around 3.5 to 3.7. We confirmed that the SaOS-2 human ostoesarcoma cell line we obtained expressed also only bone-specific ALP.

After extraction, the ALP found in U-2 OS cell were found to be identical to placental or germ cell ALP. It has a similar electrophoretic movement in the IEF gel with the placental standard prepared, which move toward the cathode and has pI value around 4.4 (Figure 2.23). Biochemical studies on the ALP extraction in U-2 OS, Sa OS-2 and the placental standard also demonstrate the ALP expressed in U-2 OS is heat-stable and sensitive to L-phenylalanine inhibition like that of placental ALP standard. Although the placental and the germ-cell ALP isozyme share many biochemical properties, they have different electrophortic movement in IEF gel (Rosendahl *et al.*, 1987). Thus, by comparing both the biochemical and electrophortic properties of ALP in U-2 OS and the placental standard, we concluded that the ALP expressed in U-2 OS ostoesarcoma cell line are placenta origin.

With both human osteosarcoma cell lines, one of them has an ectopic expression of placental ALP and the other has only BALP expression. Note should be paid to the difference between these 2 cell lines. For SaOS-2, the osteosarcoma cell is more differentiated compare with the U-2 OS cells. Moreover, the BALP expression in SaOS-2 is about 100 times higher than that of U-2 OS. U-2 OS were often refer to no expression of ALP (Manara *et. al.*,

1996). Moreover, recent research found that U-2 OS has higher ability to form solid tumor in nude mice model comparing with SaOS-2 and the transfection of the active tissue non-specific gene back to the cell line reduce its aggressiveness of the cell line (Manara *et al.*, 1996). Thus, the expression of the Regan isozyme may have a higher chance to occur in tumor with less differentiation and higher tumorgenicity.

With the finding of ectopic expression of ALP in cultured osteosarcoma cell line, we postulated that ectopic expression may also found in osteosarcoma patients. We screen for the patients' pre-treatment plasma samples to search for the ectopic expression. Pretreatment plasma samples were used for the investigation to avoid the interference from the treatment to the patients. However, all pretreatment plasma samples show only elevated plasma BALP level and liver ALP (Figure 2.28). Sometimes, patients with blood group O or B, there may find the expression of intestinal ALP in the plasma as they are active secretor of this enzyme.

The lack of ectopic expression finds in patients' plasma samples may imply:

1) There is no ectopic expression of ALP in our osteosarcoma case ; or
2) There is expression of ectopic ALP in our osteosarcoma cases but the expression is too low or it does not secrete into the body fluid.

ALP is extracted in fresh human osteosarcoma tissues obtained in the biopsy and then investigated using IEF (Figure 3.29). Results show in all 12 patients osteosarcoma tissue collected, none of them have expression of ALP isozyme other than the BALP. In ALP extracts with very high BALP level, there are addition bands found in the alkaline region of the gel with pI values above 5. This fraction is not considered as ectopic expression of ALP as the band has the same properties with the BALP isoform: it is completely destroyed in 56°C heating and is inhibited in 0.06 mM of levamisole. In fact, several authors have described the finding of this additional band (Sinha *et. al.*, 1986; Rosendahl *et. al.*, 1987). This fraction often appeared when running

sample with high level of BALP activity. The location of the fractions at pH around 5 indicates that they contain a smaller number of sialic acid residues than the bulk of bone ALP fractions.

Although ectopic expression of placental ALP was found in human osteosarcoma cell line, we cannot demonstrate the same expression in our osteosarcoma patients. The absence of ectopic expression of ALP can be explained by several postulations. The incidence of ectopic ALP expression maybe too low so with our limited samples collected, we cannot find any ectopic expression. Or ectopic expression in the cultured osteosarcoma cell line is just the differentiation change occur during the development of the cell line. There also have a chance that there is ectopic expression of ALP in our samples but the level is too low for our detection, especially after the long extraction process of ALP, this ectopic expression may be lost.

To further confirm our finding, a more sensitive method, immunohistostaining for the ectopic expression of placental ALP were employed. Monoclonal antibody against human placental ALP was used with specific affinity towards placental ALP but not other ALP isoforms. In other positive and negative controls using human placenta tissue section and human liver tissue section, we demonstrated that this antibody is specific to placental ALP and does not cross react with the liver ALP isozyme.

Both the culture human osteosarcoma cell line and the paraffin sections of the osteosarcoma tissue were examined. In the 2 human osteosarcoma cell line, we confirmed our finding on the placental ALP expression in U-2 OS osteosarcoma cell. However, in the clinical osteosarcoma tissue sections, of all 7 paraffin blocks show no ectopic expression of placental ALP. We therefore concluded that there is no ectopic expression of placental ALP in our osteosarcoma samples.

In an initial attempt to find a more specific biochemical marker for osteosarcoma, we try to explore any ectopic expression of ALP in osteosarcoma. Howevr, in all our clinical samples, both the tissue and the

plasma samples, there is no ectopic expression found. The lack of ectopic expression may be results of limitation in the number of samples collected. Even with the finding of these placental ALP expression in osteosarcoma in continuation of the project, the use of it as a biochemical marker is not a good choice due to it low incidence of expression. We therefore concluded that the search for the ectopic expression of ALP and the use of it as a biochemical marker is unsuccessful.

Chapter Five : Conclusion

5. Conclusion

5.1 Plasma bone-specific ALP as biochemical marker of osteosarcoma.

As stated in the introduction sections, biochemical chemical marker of the tumor should satisfy a number of requirements. We try to evaluate the usefulness of plasma BALP as a biochemical marker in osteosarcoma following these requirements. BALP is produced by the tumor cells and is readily detectable in body fluid. Quantitative measurement of plasma BALP level is now possible by employing an monoclonal antibody capture assay, the ALKPHASE-B®, with only very low cross influence with the LALP (5%). With these newly developed ELISA kit, the lowest possible detection level of plasma BALP is 0.7 U/L.

Plasma BALP level at admission shows significant diagnostic and prognostic value in osteosarcoma. When plasma BALP-adm was compared to the normal reference of plasma BALP level obtained in local Chinese population, it is significantly higher in osteosarcoma patients then in normal subjects with age above 12, where the highest incidence of osteosarcoma occurred.

In addition, plasma BALP-adm plays an important prognostic role in osteosarcoma. In term of long term survival of the patients, a high initial plasma BALP value indicated a much poor clinical outcomes. Patients with longer disease free survival period show significantly lower plasma BALP-adm. In addition, plasma BALP-adm can identify the patients with higher chance of local recurrence after the operative removal of the osteosarcoma. Significantly higher plasma BALP-adm value was found in the recurrence group than the non-recurrence patients. These predictions provide valuable information to the clinician so that they may highlight the so call "high risk" patients and plan for a more aggressive therapy to improve the clinical outcome of these patients.

In term of quantitative reflection of the bulk of malignancy, plasma BALP-adm direct reflects the tumor burden of the patients. We show that there is a positive correlation between the plasma BALP-adm with the tumor volume, i.e. with higher plasma BALP activity, the volume of the tumor is higher.

Most important of all, the change of plasma BALP level reflected the effectiveness of the treatment. It levels correlated well with the successfulness of the pre-operative chemotherapy. Normalization of plasma BALP occurred only after a successful chemotherapy, as indicated by the necrosis factor of the tumor, and after the surgical resection of the tumor. Plasma BALP level is also a valuable tool in follow-up period as it can detect early metastasis and thus may allow an early treatment to the relapse.

We therefore strongly suggested that the plasma BALP measurement should performed as routine analysis in every cases of suspected osteosarcoma and in the follow-up clinic.

5.2 ALP isozymes expression in osteosarcoma

Ectopic expression of placental ALP was found in human osteosarcoma cell line U-2 OS by both the biochemical studies and IEF analysis. The ALP expressed in this cell line is different from SaOS-2 cell line that is known for the sole expression of BALP. ALP expressed in U-2 OS cells show stability in 56°C and 65°C heating, L-phenylalanine sensitivity but insensitive to levamisole inhibition. Moreover, upon the separation by IEF, ALP expressed in U-2 OS shows similar band position with the placental ALP standard prepared. The ectopic expression of the placental ALP in cell line U-2 OS is further confirmed by our immunohistostaining result.

In search of any ectopic expression ALP in our osteosarcoma patients, we found that none of the 14 patients we examine show any ectopic expression of ALP in both the plasma and tissue samples extracts. Moreover,

for immunohistostaining of placental ALP by using monoclonal antibody, all of the 7 samples examined did not show any expression of this enzyme. The lack of ectopic expression may due to the insufficient samples available for analysis, or the ectopic expression in the human osteosarcoma cell line is only a derepression of gene during the adaptation period of cell line establishment.

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Appendix

ROCHE ALP IFCC reagent

4-Nitrophenyl phosphate	16 mM
Aminomethylpropanol pH 10.4	0.35 mM
HEDTA	2 mM
Magnesium acetate	2 mM
Zinc sulfate	1 mM

A-Gent® Alkaline Phosphatase Reagent

p-Nitrophenyl Phosphoric Acid, [Bis(2-amino-2-ethyl-1,3-propanediol)Salt]	18 mM
Magnesium	0.5 mM
2 amino-2-methyl-1,3-propanediol	983 mM

Phosphate Buffered Saline, PBS (pH 7.4)

NaCl	140 mM
KCl	2.7 mM
KH_2PO_4	1.5 mM
Na_2HPO_4	8.1 mM

Cell Washing Buffer (pH 7.6)

Tris-HCl	10 mM
$MgCl_2 \cdot 6H_2O$	1 mM
$ZnCl_2$	0.1 mM
NaCl	150 mM

ALP Extraction Buffer (pH 7.6)

Tris-HCl	100 mM
$MgCl_2 \cdot 6H_2O$	0.1 mM
ZnCl2	0.02 mM
Triton X-100	1% (w/v)

IEF-MIX 3.6-9.3 (Sigma I-3018)

Compound	pI value
Amyloglucosidase	3.6
Trypsin Inhibitor	4.6
β-Lactoglobulin A	5.1
Carbonic Andydrase II	5.9
Carbonic Anhydrase I	6.6
Myoglobin	6.8, 7.2
Lectin	8.2, 8.6, 8.8
Trysinogen	9.3

100µl double distilled H_2O was used for reconsitution for each bottle of marker to give a protein concentration of 2 mg/ml.

Routine Procedure for Hematoxylin and Eosin Staining.

Xylene	5 min.
Xylene	5 min.
100% Ethanol	2 min.
95% Ethanol	2 min.
85% Ethanol	2 min.
70% Ethanol	2 min.
Running tape water	1 min.
Hematoxylin	8 min.
1% Acid alcohol	1 sec
Running tape water	1 min
Scott's Water	2 min.
Running tape water	1 min
Eosin	1 min
Running tape water	1 sec
70% Ethanol	10 sec
85% Ethanol	10 sec
95% Ethanol	10 sec
95% Ethanol	10 sec
100% Ethanol	2 min.
100% Ethanol	3 min.
Xylene	5 min.
Xylene	5 min.